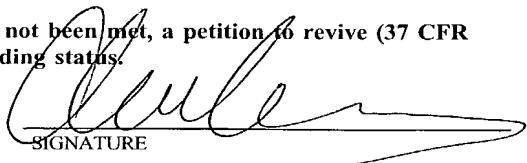


FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 43550
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/088138
INTERNATIONAL APPLICATION NO. PCT/FR00/02540	INTERNATIONAL FILING DATE 14 September 2000	PRIORITY DATE CLAIMED 17 September 1999		
TITLE OF INVENTION Novel Animal Model of Alzheimer Disease with Amyloid Plaques and Mitochondrial Dysfunctions				
APPLICANT(S) FOR DO/EO/US Saliha Moussaoui-Mrabet, Veronique Blanchard-Bregeon, Assunta Imperato, Bruno Bonici, Gunter Tremp, Christian Czech				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <ul style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2))</p> <ul style="list-style-type: none"> a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <ul style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>				
Items 11 to 20 below concern document(s) or information included:				
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Search Report</p>				

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO. IIXT/FP00/02540	ATTORNEY'S DOCKET NUMBER 43550																									
101088138		CALCULATIONS PTO USE ONLY																									
<p>21. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00</p> <p style="text-align: center;">ENTER APPROPRIATE BASIC FEE AMOUNT =</p>																											
		\$ 890.00																									
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p>		\$ 130.00																									
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>CLAIMS</th> <th>NUMBER FILED</th> <th>NUMBER EXTRA</th> <th>RATE</th> <th>\$</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>54 - 20 =</td> <td>34</td> <td>x \$18.00</td> <td>\$ 612.00</td> </tr> <tr> <td>Independent claims</td> <td>1 - 3 =</td> <td>0</td> <td>x \$84.00</td> <td>\$</td> </tr> <tr> <td colspan="2">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td></td> <td>+ \$280.00</td> <td>\$ 280.00</td> </tr> <tr> <td colspan="2" style="text-align: right;">TOTAL OF ABOVE CALCULATIONS</td> <td>=</td> <td style="text-align: right;">\$ 1912.00</td> <td></td> </tr> </tbody> </table>		CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	Total claims	54 - 20 =	34	x \$18.00	\$ 612.00	Independent claims	1 - 3 =	0	x \$84.00	\$	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$ 280.00	TOTAL OF ABOVE CALCULATIONS		=	\$ 1912.00		
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TOTAL OF ABOVE CALCULATIONS		=	\$ 1912.00																								
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +		\$																									
SUBTOTAL		= \$ 1912.00																									
<p>Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</p>		\$																									
TOTAL NATIONAL FEE		= \$ 1912.00																									
<p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</p>		\$																									
TOTAL FEES ENCLOSED		= \$ 1912.00																									
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		charged:	\$																								
<p>a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>1912.00</u> to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. <u>18-2220</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>18-2220</u>. A duplicate copy of this sheet is enclosed.</p> <p>d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>																											
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.</p> <p>SEND ALL CORRESPONDENCE TO:</p> <p style="text-align: right; margin-right: 100px;"></p> <p style="text-align: right;">SIGNATURE</p> <p style="text-align: right;">Dean H. Nakamura</p>																											
NAME																											
33,981																											
REGISTRATION NUMBER																											

10/088/138

26 November 2002

Initial Information Data Sheet

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Application Information

Title Line One::
Title Line Two::
Title Line Three::
Total Drawing Sheets::
Formal Drawings?::
Application Type::
Docket Number::

Novel Animal Model of Alzheimer Disease
with Amyloid Plaques and Mitochondrial
Dysfunction
27
No
Utility
43550

Representative Information

Registration Number:: 33,981

43550

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
Moussaoui-Mrabet et al. : Patent Art Unit: TBA
Serial No. Based on PCT/FR00/02540 : Examiner: TBA
Filed: 15 March 2002 :
For: Novel Animal Model of Alzheimer :
disease with Amyloid Plaques and :
Mitochondrial Dysfunctions :

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to prosecution on the merits, kindly amend the application as follows.

IN THE CLAIMS:

Kindly amend the claims as follows.

10. (Amended) A method for identifying compounds which can be used for treating neurodegenerative diseases comprising exposing said compounds to the animal model of any one of claims 1 to 9.

11. (Amended) Cell which is extracted from an animal model as described in accordance with any one of claims 1 to 9.

PRELIMINARY AMENDMENT
Serial No. Based on PCT/FR00/02540

12. (Amended) A method for identifying compounds which can be used for treating neurodegenerative diseases comprising exposing said compounds to the cell of claim 11.

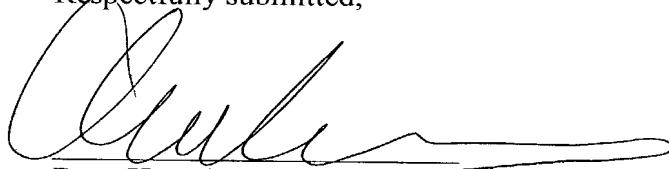
Kindly add the following new claims.

13. The method of claim 10, wherein said neurodegenerative disease is Alzheimer's disease.

14. The method of claim 12, wherein said neurodegenerative disease is Alzheimer's disease.

Favorable consideration and early indication of allowance are solicited earnestly.

Respectfully submitted,



Dean H. Nakamura
Attorney for Applicants
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Dated: 15 March 2002

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8. Animal model according to Claim 7,
characterized in that the proteins are
intramitochondrial proteins.

9. Model according to Claim 8,
5 characterized in that the proteins are the BAX and/or
cytochrome C proteins.

10. [Use of the animal model as described in
accordance with Claims 1 to 9] ^{A method} for identifying compounds
which can be used for treating neurodegenerative
10 diseases, preferably Alzheimer's disease.] Comprising exposing said compounds to
the animal model
11. Cell which is extracted from an animal ^{exposing said compounds to} of any one of
model as described in accordance with Claims 1 to 9. ^{any one of} diseases.

12. Use of a cell as described in accordance
with Claim 11 ^{A method} for identifying compounds which can be
15 used for treating neurodegenerative diseases [preferably Alzheimer's disease] Comprising exposing said
compounds to the cell of
claim 11.

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PCT/FR00/02540

NOVEL ANIMAL MODEL OF ALZHEIMER'S DISEASE

EXHIBITING BOTH AMYLOID PLAQUES AND
MITOCHONDRIAL DYSFUNCTION

The present invention relates to the field of transgenic animal models and, more specifically, to animal models of Alzheimer's disease. The invention relates to a novel animal model of Alzheimer's disease which exhibits both amyloid plaques and mitochondrial dysfunction.

10 Alzheimer's disease (AD) is a progressive neurodegenerative disease which affects a large proportion of the elderly population. This disease is characterized at the clinical level by a loss of memory and a decline in cognitive functions and, on the neuro-pathological level, by the presence in the brain of intracellular neurofibrillary deposits and extra-cellular deposits of the β -amyloid peptide ($A-\beta$) which forms the amyloid plaques (Yankner et al., 1996). In addition to these signs, there are a substantial number 15 of other abnormal changes, including impairments in the immune and inflammatory systems, as well as an impairment in mitochondrial function which can lead to an increase in oxidative stress and activation of the mechanisms of apoptosis, leading eventually to cell 20 death.

25 Amyloid plaques are in the main composed of $A-\beta$ peptides, having 40 or 42 residues, which are

generated during the proteolytic process of the β -amyloid peptide precursor protein (APP). The extracellular deposits of A- β are very specific for AD and associated disorders. They are the early and 5 invariable feature of all the forms of AD, including the familial forms (FAD). The FADs appear relatively early on (between 40 and 60 years of age) and are due to mutations in the APP gene in 5% of FAD cases (19 families) with six single or double false-sense 10 mutations; in the presenilin 1 (PS 1) gene in 50 to 70% of FAD cases (> to 50 families), with more than 40 different mutations identified to date; and in the presenilin 2 (PS 2) gene in fewer FAD cases, with 2 false-sense mutations described in 8 families (for 15 review, see Price and Sisodia, 1998). Mutations in these three genes have been demonstrated to induce changes in the proteolysis of APP, leading to overproduction of A- β and to the early appearance of pathology and symptoms which are similar to those of 20 the sporadic forms of AD.

In addition to the mutations in the APP, PS1 and PS2 genes, other factors contributing to AD have been demonstrated in man, in particular mitochondrial dysfunction (Beal, 1998). Thus, different investigative 25 approaches in individuals suffering from AD demonstrated that mitochondrial dysfunction was important in the appearance of the disease.

Thus, in accordance with a first approach, deficiencies in mitochondrial cytochrome C oxidase (COX or complex IV of the mitochondrial respiratory chain) activity have been demonstrated in the brains of human patients suffering from AD (Parker et al., 1989; Parker et al., 1994; Mutisya et al., 1994; Kish et al., 1992). Mutations of the mitochondrial COX genes have also been demonstrated in late forms of AD (Davis et al., 1996, 1997). Mitochondrial COX activity is decreased by 52% in *cybrid* cells in which the mitochondria of platelets from patients suffering from AD are fused to the cell line SH-SY5Y, which is characterized by a depletion of its mitochondrial DNA. This decrease in mitochondrial activity results in an overproduction of free radicals and an increase in the basal concentration of Ca^{2+} (Sheehan et al., 1997).

A second approach, which is just as important, demonstrated the important role which mitochondrial dysfunction plays in the cell death which is induced by apoptosis in patients suffering from AD (Su et al., 1997; Mac Gibbon et al., 1997; Tortosa et al., 1998; Nagy et al., 1997), and in particular the important role played by Bax, which has been identified in AD patient lesions (Bax being a mitochondrial protein of the Bcl-2 family which is known to induce cell death by opening the mitochondrial megachannel and inducing the mitochondria to release apoptotic

molecules, including cytochrome C, which is the substrate of COX and which activates caspases, as has been demonstrated in cultured cells, in the cytosol). In actual fact, Bax is concentrated both in the axonal structures surrounding the senile plaques (the location of bax is correlated with that of the A- β deposits in adjacent sections of the same brain at the level of the hippocampus in patients suffering from AD), and in neurons which are carrying early neurofibrillary degenerations, indicating that Bax plays a role in the formation of neurofibrillary degenerations in patients suffering from AD (MacGibbon et al., 1997; Tortosa et al., 1998; Nagy et al., 1997).

It is clearly shown in the literature that the neurotoxic properties of A- β which have been demonstrated *in vitro* are associated with the production of oxygenated free radicals (Pappola et al., 1998). However, this finding does not appear to solve the question of whether the oxidative stress is due to mitochondrial dysfunction or whether the oxidative stress might appear *in vivo* in the brain in association with the A- β deposits. This is because the findings, of which there a large number in the literature, which demonstrate and/or suggest that the neurotoxicity of A- β is mediated by oxidative stress are in fact only *in vitro* (and not *in vivo*) findings. Furthermore, the

in vivo toxicity of A- β has been called into question in many reports (Papolla et al., 1998).

While it is known that the A- β deposits are associated with mitochondrial dysfunction in human cerebral tissue, there has to date been no reported finding which demonstrates an association between the mitochondrial dysfunction and the A- β deposits in a transgenic animal model of AD. Two recent publications on transgenic animals expressing mutated APP (animals developed by Hsiao et al., 1996) reported an increase in superoxide dismutase (SOD) and hemo-oxygenase-1 (HO-1), and an increase in lipid peroxidation and hydroxynonenal (HNE) in association with A- β deposits (Smith et al., 1998; Papolla et al., 1998). However, all these markers are oxidative stress markers which are situated downstream of the mitochondrial dysfunction and are not specific for this dysfunction. Furthermore, some of these markers used in the previously described studies, i.e. SOD and HO-1, are not oxidizing agents which induce neuronal death but are, on the contrary, known as being antioxidants which are involved in the defence mechanisms which are brought into play in reducing oxidative damage and cell death.

As far as the presenilins are concerned, mutations of PS1 have been reported to induce apoptosis in cultured cells and *in vivo* in the brains of

transgenic mice expressing mutated PS1 (Chiu et al., 1999). However, this does not show that the apoptosis induced by the PS1 mutations is mediated by mitochondrial dysfunction. This is because the 5 apoptosis can be either the consequence of mitochondrial dysfunction or the consequence of other mechanisms of cell death which are not necessarily involved in the mitochondrial dysfunction: for example, it has been shown that inhibition of the protease 10 activity of interleukin-converting enzyme 1- β (ICE) only prevents Bax-induced changes which are downstream of the mitochondria (degradation of DNA, for example) but has no effect on mitochondrial changes such as the loss of the mitochondrial membrane potential and the 15 production of free radicals (Xiang et al., 1996). Recent in-vitro studies have demonstrated that the PS1 mutations are involved in the overproduction of free radicals, in the impairment of calcium homeostasis and in the loss of mitochondrial membrane potential (Begley 20 et al., 1999; Guo et al., 1996, 1997, 1998). However, these studies do not show any association between the mitochondrial dysfunction and the A- β deposits in transgenic mice expressing mutated PS1 since the transgenic mice used in these studies for preparing 25 synaptosomes do not develop A- β deposits. There is therefore no evidence in the literature which shows an association between mitochondrial dysfunction and A- β

deposits *in vivo* in the brains of transgenic mice which are expressing mutated PS1 (Chui et al., 1999) or mutated APP (Hsiao et al., 1996; Irizzary et al., 1997a, 1997b; Johnson-Wood et al., 1997), or else 5 coexpressing mutated APP and mutated PS1 (Borchelt et al., 1997; Holcomb et al., 1998).

The present invention therefore results from the demonstration, for the first time, that amyloid deposits are associated with mitochondrial dysfunction 10 *in vivo* in the brains of transgenic animals.

Furthermore, the invention also results from a search for a novel animal model of AD which is more representative and which reproduces the neuropathology which is encountered in man.

15 The invention firstly relates, therefore, to a transgenic animal model of Alzheimer's disease which exhibits both amyloid plaques and mitochondrial dysfunction. Advantageously, it coexpresses the β -amyloid peptide precursor (APP) and a presenilin, 20 preferably PS₁.

A transgenic animal is understood as being any non-human animal whose genome has been modified. The modification of the genome can result from an alteration or modification of one or more genes by 25 knock-in or knock-out. This modification can be due to the action of conventional mutagenic or altering agents

or else brought about by site-directed mutagenesis, as described in Materials and Methods.

The modification of the genome can also result from the insertion of (a) gene(s) or the 5 replacement of (a) gene(s) in its (their) wild-type or mutated form.

The modifications of the genome are advantageously carried out on reproductive stem cells, advantageously on their pronuclei.

10 Within the context of the present invention, the animal model is advantageously a mammal. In particular, it can be a mouse, a rat or a rabbit which is obtained using the conventional techniques of transgenesis. By way of an example illustrating one of 15 the procedures of transgenesis, mention may be made of the method of microinjecting an expression cassette which comprises the modified genes in the two fertilized pronuclei, as described in Materials and Methods.

20 In this respect, the animal model of the invention is obtained by injecting an expression cassette which comprises a nucleic acid. This nucleic acid is preferably a DNA which can be a genomic DNA (gDNA) or a complementary DNA (cDNA).

25 Within the context of the model of the invention, the DNA encodes any gene which is able to play a part in the process of establishing the AD.

Advantageously, the gene encoded by the DNA is involved in the mechanism of producing the A- β peptide in its amyloidogenic form.

In particular, the DNA encodes mutated forms 5 of APP and/or presenilins, in particular PS1, such that the cells of the animal model coexpress the two mutated proteins.

The mutations in the APP gene can be one of the various mutations which have to date been described 10 in the literature. Preferably, the mutations in the APP gene are selected from the "Swedish" (S), "London" (L) and "Dutch" (D) mutations, taken on their own or in combination.

These mutations are amply described in the 15 literature and are generally characterized by the following modifications:

Nature and position	Swedish mutation	Dutch mutation	London mutation
in relation to APP770	K 670 N and M 671 L	E 693 Q and/or A 692 G	V 717 I
in relation to APP751	K 651 N and M 652 L	E 674 Q and/or A 673 G	V 698 I
in relation to APP695	K 595 N and	E 618 Q and/or	V 642 I

	M 596 L	A 617 G	
in relation to the A- β peptide (A42)		E 22 Q and/or A 21 G	V 46 I

The London mutation also comprises all the substitutions other than with isoleucine which are located in position 717 with reference to APP770, such 5 as the V 717 G and V 717 F mutations.

It is understood that the APP which can be used within the context of the invention can be in different isoforms, in particular in the 695, 751 and 770 forms, or in a truncated form such as the APP99 10 isoform.

The mutations in the PS1 gene can be one of the 40 mutations described to date in the literature. Preferably, the mutations in the PS1 gene are selected from the M146L, A246E, C410Y, H163R, L226V, L235P, etc. 15 mutations, taken on their own or in combination.

The M146L mutation is preferred for preparing a model according to the invention.

Within the context of the model of the invention, the DNA is placed under the control of 20 sequences which enable it to be expressed, in particular transcription promoter sequences.

Promoter sequences which may be mentioned are, very particularly, the HMG promoter (Gautier

et al., 1989), and also the PDGF promoter (Sasahan et al., 1991), the Thy-1 promoter (Lüthi et al.,) and the Prion gene promoter (Scott et al., 1992).

According to a particularly favourable embodiment of the invention, the animal model comprises the APP gene possessing the S, D and L mutations, with this gene being placed under the control of the PDGF promoter, and the PS1 gene possessing the M146 L mutation, with this gene being placed under the control 10 of the HMG promoter.

The animal model according to the invention is very advantageous since it is a model which is very representative of AD. This is because this model develops amyloid plaques from the age of 6 months, 15 which makes the time required for breeding the animals very short, and coexpresses mutated APP and PS1 proteins at levels which are clearly higher than the endogenous levels; at least 3 to 5 times and 2 to 3 times the endogenous levels of APP and PS1, 20 respectively.

Thus, the results described in the examples show that the transgenic mouse which is coexpressing mutated APP and mutated PS1 develops a neuropathology of the Alzheimer's disease type; that is, it exhibits 25 A- β deposits having a fibrillary conformation, neurodegenerative changes of the abnormal axonal structure type, and activation of central nervous

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system cells of the inflammatory type such as astrocytes.

Particularly favourably, this model exhibits mitochondrial dysfunction, which has also been shown in 5 patients suffering from AD, in addition to the amyloid plaques.

The results described in the examples show the involvement of mitochondrial dysfunction in the neuropathology of these transgenic AD mice. In relation 10 to the findings which have been published to date in the mitochondrial and Alzheimer's disease fields, these results represent the first demonstration, by means of studying two important mitochondrial markers, i.e. Bax and cytochrome C, that mitochondrial dysfunction occurs 15 in the brains of the transgenic AD mice of the invention. Finally, these results demonstrate that Bax and cytochrome C are expressed in axonal structures which are intimately associated with the A- β deposits 20 in the brain of the transgenic AD mouse in the same way as in the brains of AD patients.

In the present invention, the mitochondrial dysfunction is understood as being an alteration, a modification, an overexpression or a inhibition of the expression of the mitochondrial proteins. These 25 proteins, which preferably have a subcellular intramitochondrial location, include the proapoptotic proteins of the Bcl-2 family, such as Bax, Bak and Bad,

and the antiapoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-xL, or any other mitochondrial protein which does not belong to the Bcl-2 family and which plays a role in apoptosis, such as cytochrome C and AIF, or else proteins which have recently been recorded as being located in the mitochondria and which can play a role in apoptosis, such as Aralar or BMCP1.

Mitochondrial proteins whose expression is modified and which may preferably be mentioned are, in particular, the Bax and/or cytochrome C proteins.

The present invention also relates to the use of the animal model, as previously described, for identifying compounds which can be used for treating neurodegenerative diseases, preferably Alzheimer's disease.

Thus, as a result of its advantageous properties, which very faithfully reproduce the characteristics of AD, this model can be used, by contrast with the known models, to identify compounds which are particularly well suited for treating AD, in particular AD as described in man.

These compounds can be chemical molecules, peptide or protein molecules, antibodies or chimeric molecules, and also antisense DNAs or ribozymes.

The compounds which have been identified can be used as medicaments, as such or in combination with a pharmaceutically acceptable excipient in order to

obtain a pharmaceutical composition. The excipients can, in particular, be sterile, isotonic saline (monosodium phosphate, disodium phosphate, sodium chloride, potassium chloride, calcium chloride or 5 magnesium chloride, etc., or mixtures of such salts) solutions, or dry, in particular lyophilized, compositions which enable injectable solutions to be constituted by the addition, as the case may be, of sterilized water or physiological saline. The 10 injections can be effected by the stereotactic, topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, etc. route.

The identification of the previously 15 described compounds is based on bringing the animal model of the invention into contact, in particular by means of an administration, such as an injection, with a compound or a mixture of compounds which is/are assumed to have an action and then measuring the 20 effect(s) of the compounds, in particular at the cerebral level of the model, on various biochemical and/or histological changes, for example those described in the Methods and Results sections, including the level of production of the A- β deposits, 25 the changes linked to neurodegeneration, alteration in the expression of mitochondrial molecules, etc.

The invention also relates to a cell which has been extracted from the previously described animal model and to its use for identifying compounds which can be used for treating neurodegenerative diseases, 5 preferably Alzheimer's disease.

The identification of previously described compounds is based on bringing cells which have been extracted from the animal model of the invention into contact with a compound or a mixture of compounds which 10 is/are assumed to have an action and then measuring the effect(s) of the compounds, at the level of the whole cells, in cell homogenates, or on a subcellular fraction, on various parameters such as cell death, production of the A- β peptide, and mitochondrial 15 activity (production of free radicals, respiratory chain, mitochondrial potential, etc.).

The results described in the examples show the advantages of the model of the invention and clearly support using this transgenic model for 20 developing therapeutic strategies such as, in particular, mitochondrial agents which resist mitochondrial dysfunction and the neuronal death which is induced by mitochondrial dysfunction.

The present invention will be described in 25 more detail with the aid of the examples, which follow and which should be regarded as being illustrative and not limiting.

LEGEND TO TABLE I: the table recapitulates the results which were obtained in the double transgenic mouse model exhibiting A- β deposits and markers of apoptosis 5 which is mediated by mitochondrial dysfunction in axonal structures.

LEDEND TO THE FIGURES

Figure 1

Comparison of the levels of expression of 10 APP, of the β -secretase fragment and of A- β in various transgenic mouse lines: 1 (AA LD2 (B6)), 2 (APP LD2 (FVB)), 3 (NT), 4 (Thy-1 Kozak APP₇₅₁ SL (28)), 5 (Thy-1 Kozak APP₇₅₁ SL (26)), 6 (Thy APP₇₅₁ SDL (1001)), 7 (PDGF APP₆₉₅ SDL (46)), 8 (HMG APP SDL 20 (76)).

15

Figure 2A

Use of the antibody WO-2, which is specifically directed against the human form of A- β , 20 for analyzing the expression of APP, of the β -secretase 12kDa fragment and of A- β in homogenates of brains of transgenic mice which are of different ages and which carry a single or double mutation. While the age of the transgenic mice does not alter the level at which APP 25 is expressed, it leads to pronounced accumulation of A- β .

Figure 2B

Analysis of the expression of human M146L
PS1.

5

Figure 3

Plate illustrating the expression of human APP in the hippocampus and cortex of transgenic mice carrying a single mutation (APP mutant) (b1, b2) or a double mutation (APP/PS1 mutant) (c1, c2). Note the high level of APP expression in the hippocampal (b1, c1) and cortical (b2, c2) neurons of these two lines of transgenic mice and the very low, if not undetectable, level of APP expression in the corresponding cerebral regions of the nontransgenic control animals (a1, a2). In the same way, the expression of human PS1 is evident in the same cerebral regions in the transgenic mice carrying a single mutation (PS1 mutant) (e1, e2) or a double mutation (APP/PS1 mutant) (f1, f2) and is undetectable in nontransgenic control animals (d1, d2).

Figures 4 and 5

25

Illustration of the A- β deposits in the brains of transgenic mice which carry a double mutation

(APP/PS1 mutant) and which are 6 months (Fig. 4) and 12 months (Fig. 5) of age. Immunohistochemical detection using several antibodies directed specifically against different epitopes of the A- β peptide: antibodies 6E10 5 for A β ₁₋₁₇ (Figure 4A and 5A), Dako for A β ₈₋₁₇ (Figure 4B and 5B), 4G8 for A β ₁₇₋₂₄ (Figure 4C and 5C), QCB for A β ₁₋₄₂ (Figure 4D and 5D) and FCA18 for total A β (Figure 4E and 5E).

10 **Figure 6**

Pictures depicting histological sections, stained with thioflavin S (Figure 6A) and Congo red (Figure 6B) (staining green and red, respectively), at 15 the level of the hippocampal formation in transgenic mice which carry a double mutation (APP/PS1 mutant) and which are 12 months of age. Demonstration of the fibrillary conformation of the A- β deposits in the brains of these mice. Note the variable morphology of 20 the A- β deposits; spherical (arrows) or irregular (arrow heads) forms in the transgenic mice carrying a double mutation (APP/PS1 mutant).

Figure 7

25 Progression of the A- β deposits, which were immunolabelled with the anti-A β antibody 4G8, in the transgenic mouse carrying a double mutation (APP/PS1

mutant). Note that the density of the A- β deposits is much more pronounced at 12 months of age (C1, C2, C3) as compared with 9 and 6 months of age (B1, B2, B3 and A1, A2, A3, respectively). Also note that the A- β 5 deposits are in the main located in a restricted cerebral region, principally the subiculum, in the young mouse (A1-A2), whereas they are present in the whole of the hippocampal formation and in cortical regions in the 12-month-old mouse (C1-C2). Figures A2, 10 B2, C2 and A3, B3, C3 depict a higher magnification of the regions demarcated by a black frame in Figures A1, B1, C1 and Figures A2, B2, C2, respectively. Hi: hippocampus, Ctx: cortex.

15 **Figure 8**

Number of A- β deposits, which were immunolabelled with the anti-A β antibody 4G8, in 6 μ m thick hemibrain sections (Bregma rostrocaudal level - 20 3.4 of Franklin and Paxinos' Stereotactic Atlas) from the double transgenic APP₆₉₅ SDL X PS1 M1476 L mouse, plotted against age (6, 9 and 12 months); the deposits were quantified using an image analysis system coupled to a colour camera and a microscope (Q600, LEICA).

Figure 9

Regional distribution of the A- β deposits in the brain of a transgenic mouse which carries a double mutation (APP/PS1 mutant) and was 12 months of age. 25 μm sections corresponding to 6 representative levels of the rostrocaudal axis of the mouse brain were immunolabelled with the anti-A β Ab 4G8. Note the very high number of A- β deposits in the hippocampal formation and all the cortical regions.

Figure 10

Quantification, in all the 12-month-old transgenic mice carrying a double mutation (APP/PS1 mutant), of the A- β load, as visualized by A β immunohistochemistry (antibody 4G8) carried out on 25 μm thick hemibrain sections (Bregma rostrocaudal level - 3.4). Note the A- β load, which reaches more than 3 and 1% of the total surface of the hippocampus and the cortex (Figure 10B), respectively, and more than 9 and 5%, respectively, in the richest regions of these two cerebral structures (Figure 10C). On the other hand, the A- β load is less than 0.5% in the subcortical regions.

1: Dorsal cortex, comprising, in particular, the primary visual cortex and the auditory cortex

2: Ventral cortex, comprising, in
5 particular, the ectorhinal cortex and the entorhinal cortex

3: Hippocampal formation

4: Remainder of the hemibrain (subcortical structures)

10

Figure 11

Figure 11A shows the presence of APP-immunoreactive axonal structures (arrow heads in 4) in the hippocampus of transgenic mice carrying a double mutation (APP/PS1 mutant) (4) and their absence in the hippocampus of nontransgenic control mice (1) and of transgenic mice carrying a single APP mutation (APP mutant) (2) or PS1 mutation (PS1 mutant) (3). In the 20 transgenic mice carrying a double mutation (APP/PS1 mutant), the APP-immunoreactive axonal structures are also present in all the cortical regions as Figure 11B shows, including the entorhinal cortex (a) and the cingulate cortex (d), in the dentate gyrus and in the 25 CA1 and CA3 hippocampal regions (b, c). The regions demarcated by a black frame in Figures b, b1 and b3 are visualized at greater magnification in b1, in b2 and

b3, and in b4, respectively. Also note the very high level of expression of the human APP protein in some neuronal cell bodies (arrows in b2). The arrow in 1, 2, 3 and 4 in each case indicates the orientation of the head of the dentate gyrus.

Figure 12

Figure 12A shows the presence of PS1-
10 immunoreactive axonal structures (arrow heads in 4) in
the hippocampus of transgenic mice carrying a double
mutation (APP/PS1 mutant) and their absence in the
hippocampus of nontransgenic control mice and of
transgenic mice carrying a single APP mutation (APP
15 mutant) or PS1 mutation (PS1 mutant) (3). In transgenic
mice carrying a double mutation (APP/PS1 mutant), the
PS1-immunoreactive axonal structures are also present
in all the cortical regions as Figure 12B shows,
including the entorhinal cortex (a) and the cingulate
20 cortex (d), in the dentate gyrus and in the hippocampal
regions CA1 and CA3 (b, c, e). The regions demarcated
by a black frame in Figures c, d and e are visualized
at great magnification in c', in d' and in e',
respectively. Also note the very high level of
25 expression of the human PS1 protein in some neuronal
cell bodies in e' and d' (arrows).

Figure 13

This figure shows the presence of delta catenin-immunoreactive axonal structures in the hippocampus (Hi) and the cortex (Ctx) of transgenic mice carrying a double mutation (APP/PS1 mutant) (b1, b2) and their absence in the same cerebral structures in nontransgenic control mice (a1, a2). The regions demarcated by a black frame in Figures b1 and b2 are visualized at greater magnification in bla, b1b and in b2a, b2b, respectively. Note that, just like the APP-immunoreactive and PS1-immunoreactive axonal structures, the delta catenin-immunoreactive axonal structures appear to be grouped in plaques of varying size, i.e. small, medium or large.

Figure 14

Synaptophysin-immunoreactive axonal structures in transgenic mice carrying a double mutation (APP/PS1 mutant) (Figure 14A) and in humans suffering from AD (Figure 14B). Note that, in doubly immunolabelled brain sections, the synaptophysin-immunoreactive axonal structures (brown immunolabelling) tightly surround the A- β deposits (blue immunolabelling) in both cases.

Figure 15

SMI-immunoreactive phosphorylated neurofilaments in transgenic mice carrying a double mutation (APP/PS1 mutant) and in humans suffering from AD. Note that, in doubly immunolabelled brain sections, the SMI-immunoreactive phosphorylated neurofilaments (brown immunolabelling) tightly surround the A- β deposits (blue immunolabelling) in both cases.

10

Figure 16

tau-1 Immunoreactivity (brown immunolabelling) concentrated in axonal structures including dystrophic axons which are located around or inside the A- β deposits (blue immunolabelling) and neuronal cell bodies (arrow heads) in the brains of transgenic mice carrying a double mutation (APP/PS1 mutant). Neither dystrophic axons nor cell bodies which are immuno-reactive for the tau-1 protein are present in the non-transgenic control mice.

Figure 17

Activated, GFAP-immunoreactive astrocytes (brown immunolabelling) which are present in transgenic mice carrying a double mutation (APP/PS1 mutant)

(Figure 17A) and in humans suffering from AD (Figure 17B). Note that, in doubly immunolabelled brain sections, the activated astrocytes surround some A- β deposits (blue immunolabelling) in both cases.

5

Figure 18

Figures 18A to 18H show Bax expression in the neurons of the dentate gyrus (Figure 18A, Figure 18E), 10 the CA1 (Figure 18C, Figure 18G) and CA3 hippocampal regions (Figure 18B, Figure 18F) and the entorhinal cortex (Figure 18D, Figure 18H) of a nontransgenic control mouse (Figure 18A to Figure 18D) and of a transgenic mouse carrying a double mutation (APP/PS1 15 mutant) (Figure 18E to Figure 18H). Note that, while Bax-immunoreactive axonal structures (arrow heads) are present in the doubly transgenic mouse, they are absent from the nontransgenic control mouse.

Figures 18I to 18L show a double 20 immunolabelling which makes it possible to visualize Bax expression (in brown) and the A- β deposits (in blue) in one and the same section of the dentate gyrus (Figures 18K and 18L) and the CA3 hippocampal region (Figures 18I and 18J) of transgenic mice carrying a 25 double mutation (APP/PS1 mutant). Note that the Bax immunoreactivity is concentrated in abnormal axonal structures such as dystrophic axons which are

intimately associated with the A- β deposits (arrows). The Bax immunoreactivity is also concentrated in some abnormal cell bodies which appear to be strongly immunolabelled at the level of their perinuclear cytoplasm and which resemble so-called "dark" neurons (black arrow heads). Bax immunoreactivity is also apparent in some glial cells which are reminiscent of activated astrocytes (white arrow heads).

10 **Figure 19**

Bax-immunoreactive axonal structures in the frontal cortex from a control human subject (Figure 19A) and from a patient suffering from AD (Figure 19B to 19F). Note, in the AD patient but not in the control subject, the presence of Bax-immuno-reactive axonal structures (brown immunolabelling) such as dystrophic axons (arrows), some of which appear to be intimately linked to the A- β deposits (blue immunolabelling) and abnormal cell bodies which exhibit very intense labelling areas in the cytoplasm and the proximal prolongations, thereby resembling neurofibrillary degenerations (arrow heads).

Figure 20

Figures 20A and 20B: Pictures depicting cytochrome C expression in the CA1 hippocampal region from nontransgenic control mice (Figure 20A) and transgenic mice (Figure 20B) carrying a double mutation (APP/PS1 mutant). Note the presence of cytochrome C-immunoreactive axonal structures (arrows) in the double-transgenic mouse and their absence from the nontransgenic control mouse.

Figures 20C to 20H: pictures depicting a double immunolabelling which makes it possible to visualize cytochrome C expression (in brown) and the A- β deposits (in blue) on one and the same section of the dentate gyrus, hippocampal regions CA1 and CA3 and the cingulate cortex from transgenic mice carrying a double mutation (APP/PS1 mutant). Note that the cytochrome C immunoreactivity is concentrated in abnormal axonal structures such as dystrophic axons which are intimately associated with the A- β deposits (arrows in b, c, d, e and f). The cytochrome C immunoreactivity is also concentrated in certain abnormal cell bodies which appear to be strongly immunolabelled at the level of their perinuclear cytoplasm (arrow heads in c, d and f). The high-magnification pictures in g and h demonstrate the intimate association of the cytochrome C-immunoreactive

cell structures with the A- β deposits (arrows in g and h).

Figure 21

5

Cytochrome C-immunoreactive axonal structures in the frontal cortex from a control human subject (Figure 21A) and from a patient suffering from AD (Figures 21B to 21F). Note the presence, in the AD 10 patient and not in the control subject, of cytochrome C-immunoreactive axonal structures (brown immunolabelling) such as dystrophic axons (arrows), some of which appear to be intimately linked with the A- β deposits (blue immunolabelling) and abnormal cell 15 bodies which exhibit very intense labelling areas in the cytoplasm and in the proximal prolongations, thereby resembling neurofibrillary degenerations (arrow heads).

20 **Figure 22**

Double immunofluorescent labelling making it possible to visualize Bax expression, by means of red rhodamine fluorescence (Figures 22A to C), and the 25 expression of APP, PS1 or SMI, by means of green fluorescein fluorescence (Figures 22D to F respectively), in one and the same brain section from

transgenic mice carrying a double mutation (APP/PS1 mutant). Note the presence of Bax and APP, and also of Bax and PS1, within the same neuronal structures (colocation in the same neurons and plaques), and, in 5 the case of control, the colocation of the Bax and SMI immunofluorescences within or around the plaques and not in the cell bodies. The double immunofluorescent labelling (Figures 22G to 22H) demonstrates the colocation of cytochrome C and delta-catenin in a 10 substantial number of neuronal structures (but not in all of them) in the brains of the double transgenic mice. Pictures 22I and 22J demonstrate the double 15 immunofluorescent labelling of Bax and APP and their colocation in the axonal structures within or around the plaques in the cerebral tissue of a human patient suffering from AD.

Figure 23

20 Double immunofluorescent labelling making it possible to visualize Bax expression, by means of red CY3 fluorescence (Figures 23A to 23C), and expression of APP, PS1 or cytochrome C, by means of green fluorescein fluorescence (Figures 23D, 23E and 23F 25 respectively), in one and the same brain section from transgenic mice carrying a double mutation (APP/PS1

mutant). Confirmation of the colocation of Bax and APP, of Bax and PS1 and of Bax and cytochrome C.

A/Materials and Methods

5

1. PS1 mutagenesis

Human PS1 was mutagenized using a Sculptor^{TM+} (Amersham, France) *in-vitro* mutagenesis system. The 10 PS1-encoding region, including a Kozak consensus motif, was subcloned into the Bluscript vector (Stratagene), and the mutations were introduced in accordance with the protocol supplied by the manufacturer by using oligonucleotides which contained the desired mutation. 15 The mutated sequences were verified by sequence analysis.

2. Generation and identification of the PS1 M146L transgenic mice

20

In order to construct the transgene, the cDNA encoding the mutated human PS1 was subcloned between the SmaI and BamHI restriction sites of the polylinker of the transgenic expression vector HMG (Czech et al., 25 1997). For microinjecting, the vector sequences were removed by restricting with the enzyme NotI, and the fragment containing the expression cassette was

purified by gel electrophoresis. The purified fragment was diluted in 10 mM Tris-HCl (pH 7.4) 0.1 mM EDTA to a final concentration of 2.5 ng/ μ l and injected into one of the two pronuclei of fertilized mouse embryos. The 5 surviving embryos were immediately transplanted into the oviduct of adoptive (pseudopregnant) mothers. The presence of the transgene in the neonates was determined either by PCR or by performing a Southern analysis. The PCR was carried out using oligomers 10 corresponding to human PS1 (5'-TAA TTG GTC CAT AAA AGG C- 3'; 5'-GCA CAG AAA GGG AGT CAC AAG-3'), thereby amplifying a fragment of 550 bp in size. In the case of the Southern blot, a 1.2 kb PstI-SalI fragment from the first intron from the HMG expression cassette, which 15 fragment was radiolabelled with alphaP-32-dCTP, was used as a probe for detecting the transgene and the endogenous HMG gene, as an internal control. Taken overall, these analyses are able to exclude the possibility of there having been any major 20 rearrangement in, or deletions of, the transgene in the founders and their progeny.

3. Constructing vectors for expressing the APP transgene

The plasmid containing the PDGF promoter expression cassette was linearized, using the restriction endonuclease *Sna BI* in accordance with standard procedures (Ausubel et al.; Current Protocols in Mol. Biol.), in order to generate a blunt-ended cut for the purpose of subcloning the APP cDNA.

The mutagenesis of APP was described previously (Czech et al. 1997). The cDNA encoding human APP₆₉₅SDL was excised from the cloning plasmid using the restriction endonucleases *SmaI* and *Cla*. The cohesive ends generated by *Cla*I were rendered blunt-ended by treating them with the DNA polymerase Klenow fragment. The identity and integrity of the PDGF APP₆₉₅SDL construct was verified by analyzing the restriction pattern, and the junctions between the fused DNA fragments were checked by partial sequencing.

A plasmid preparation kit (Qiagen) was used for preparing the supercoiled DNA. The complete transgene was purified as described above by digesting with the restriction enzyme *NotI* and separating the transgene fragment by means of electrophoresis. The aliquots to be used for the microinjection were dialyzed against a TE buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA) on a floating filter (Millipore; membrane type: VS; 0.025 µm) and then filtered (Spin-X; Costar; polyacetate membrane; 0.22 µm). The DNA was diluted to

a final concentration of 1-2 ng/ μ l for the microinjection.

3.2 APP₇₅₁SDL

5

The mutagenesis of the APP was described previously (Czech et al. 1997), and the mutated APP sequences were introduced into the APP₇₅₁ cDNA by inserting the exon-8-containing Sma I/Bgl II APP fragment into the Bluescript vector containing the mutations. In order to generate the transgenic expression construct APP₇₅₁SDL Thy-1, the APP cDNA, from the Sma I (-95) site to the Cla I (2699) site, was cloned into a modified pBluescript vector containing 10 SalI sites on each side of the insertion site. The vector was digested with Sal I and the insert was cloned into the murine Thy-1 vector using the Xho I site (Lüthi et al. J. Neuroscience 17, 4688-4699). The correct orientation was verified by restriction 15 analysis and the construct was sequenced at the ligation sites. For microinjecting, the cassette was linearized by Not I-Pvu I restriction and the fragment 20 containing the transgene was then purified.

25 3.3 APP₇₅₁Kozak SL

The mutagenesis of the APP was described previously (Czech et al. 1997), and the mutated APP sequences were introduced into the APP₇₅₁ cDNA by inserting the exon-8-containing Sma I/Bgl II APP fragment into the Bluescript vector containing the mutations. In order to optimize the translation initiation site of the APP, an optimized Kozak consensus sequence was introduced by site-directed mutagenesis carried out by means of PCR. For the PCR, 5 the oligonucleotide combination was as follows: sense oligo (initiation region): ccc ggg tcc acc atg ctg ccc ggt ttg g (Kozak sequence underlined), antisense oligo: ttc agg gta gac ttc ttg gc. The PCR product was cloned into pCR2 (Invitrogen, France), after which it was 10 sequenced and then subcloned into the APP₇₅₁SL cDNA-containing Bluescript vector using Sma I and Acc I, thereby deleting the 5' UTR of the APP and introducing 15 the Kozak consensus sequence. In order to generate the transgenic APP₇₅₁ Kozak SL Thy-1 construct, the above APP cDNA, extended in 3' to Cla I (2699) was subcloned 20 into a modified Bluescript vector containing two Sal I sites on each side of the insert. The vector was digested with Sal I and the insert was cloned into the murine Thy-1 vector using the Xho I site (Lüthi et al. 25 J. Neuroscience 17, 4688-4699). The correct orientation was verified by restriction analysis, and the construct was sequenced at the ligation sites. For

microinjecting, the cassette was linearized by Not I-Pvu I restriction and the fragment containing the transgene was then purified.

5 3.4 Generating transgenic animals

The transgenic animals were obtained and identified using already described standard procedures (e.g. "Manipulating the Mouse Embryo"; Hogan et al. CSH 10 Press; Cold Spring Harbor, N.Y.).

4 Western blot analysis

Cerebral tissue from transgenic mice and 15 nontransgenic control mice (littermates) was homogenized, on ice, in a 0.32 M sucrose solution containing protease inhibitors (CompleteTM, Boehringer-Mannheim, Germany). The cell debris was removed by centrifuging at 1500 g for 5 min and at 4°C. The 20 protein concentration in the supernatant was measured using the BCA protein test (Pierce, USA). For detecting PSI, 25 µg of protein extract were incubated, at 56°C for 20 min, in Laemmli loading buffer containing 8 mM urea and 50 mM dithiothreitol. For detecting APP and 25 A-β, 25 µg of protein extract were denatured, at 95°C for 10 min, in 30 µl of standard Laemmli loading buffer. The proteins were fractionated by

polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the proteins to a nitrocellulose filter (Amersham, France), the filter was warmed in PBS for 5 min in order to increase sensitivity and immediately 5 saturated for 1 h with 5% (w/V) skimmed milk powder in TBST 850 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.05% (V/V) Tween 20, and then incubated overnight at 4°C together with the primary Ab in TBST buffer alone. The binding of the Ab was detected using a horseradish-peroxidase-10 conjugated anti-IgG-Ab (Amersham, France), followed by a chemi-luminescence detection system (Amersham, France), in accordance with the manufacturer's instructions. The primary Ab MAB1563 (Chemicon, USA) was used, at 1/10,000 dilution, for detecting PS1; the 15 antibody WO-2 was used, at a concentration of 0.1 µg/ml, for detecting APP and A-β.

5 Antibodies used for the immunohistochemistry

20 The following primary antibodies (Abs) were used:

mouse anti-APP monoclonal Ab (1:100; 22C11,
Boehringer)

biotinylated mouse anti-A β ₁₇₋₂₄ monoclonal Ab
25 (1:200, clone 4G8, Senetek)

biotinylated mouse anti-A β ₁₋₁₇ monoclonal Ab
(1:200, clone 6E10, Senetek)

mouse anti- $\text{A}\beta_{8-17}$ monoclonal Ab (1:100, clone
6F/3D, Dako)

rabbit anti- $\text{A}\beta_{1-42}$ polyclonal Ab (1:300, QCB)

rabbit anti- $\text{A}\beta_{\text{total}}$ polyclonal Ab (1:1000, FCA18 from
5 Checler)

rat anti-PS1 monoclonal Ab (1:50, Chemicon)

rabbit anti-delta catenin polyclonal Ab (1:2000, RPR)

rabbit anti-GFAP polyclonal Ab (1:3000, Dako)

rabbit anti-synaptophysin polyclonal Ab
10 (1:100, Dako)

mouse phosphorylated anti-neurofilaments
(PNFs) monoclonal Ab (SMI 312, 1:100, Sternberger)

mouse anti-Tau-1 (Tau, microtubule-associated
protein) monoclonal Ab (5 µg/ml, Boehringer, Mannheim)

rabbit anti-Bax polyclonal Ab (P19, 1:300,
15 Santa Cruz)

mouse anti-cytochrome C monoclonal Ab (clone
7H8.2C12, 1:200, Pharmingen)

The secondary antibodies (1:400, Vector)
20 which were used were mouse anti-IgG Abs (H + L), in the
case of experiments involving the use of mouse primary
Abs, and rabbit anti-IgG Abs or rat anti-IgG Abs
(H + L), in the case of the experiments involving the
use of primary rabbit and rat Abs, respectively. In
25 some immunofluorescent labelling experiments, the
secondary Ab employed was a rabbit anti-IgG Ab which
was conjugated to the fluorochrome CY3 (1/400, Vector).

6 Manipulating the animals

The animals were housed under controlled
5 temperature and humidity conditions and subjected to a
12 h day/12 h night cycle (light 7:00 EST). The animals
had free access to food and water. The experiments on
these animals were carried out with the agreement of
the Rhône Poulenc Rorer Ethical Committee on the care
10 and use of animals, in conformity with the standards of
the "Guide for the care and use of laboratory animals"
(National Research Council ILAR) and while respecting
the French regulations and the EEC directive.

The animals used for the neurohistopathology
15 studies are listed in the following table:

	Nontransgenic mice (littermates)	Single-transgenic mice PDGF APP ₆₉₅ SDL	Single-transgenic mice HMG PS1M146L	Double-transgenic mice PDGF APP ₆₉₅ SDL X HMGPS1M146L
Age (months)	6 9 12 15 18 6 9 12 15 18 6 9 12 15 18 6 9 12 15			
Number	1 5 4 6 2 4 2 2 4 - 1 2 9 5 7 4 5 5 -			

7 Neurohistopathology**7.1 Preparing the cerebral tissue**

5 The mice were deeply anaesthetized (Pentobarbital: 60 mg/ml/kg i.p., Ketamine: 40 mg/ml/kg i.p.) and then perfused via the heart with physiological serum and then paraformaldehyde (4% in PBS). The brains were then removed and subsequently 10 post-fixed, at 4°C for 24 h, in the same fixing solution. After fixing, the brains were separated into right and left hemibrains, with the latter then being subjected to the standard protocol of embedding in paraffin.

15 The paraffin-embedded left hemibrains from the transgenic and non-transgenic mice, and also blocks of postmortem human brain tissue (frontal cortex) from a patient suffering from AD and a control subject (supplied by Dr J.P. Brion, Belgium), were sectioned, 20 at a thickness of 6 µm (serial sections), using a microtome (LEICA RM 2155, France). The tissue blocks corresponding to the right hemibrains from the transgenic and nontransgenic mice were sectioned to a thickness of 25 µm.

25 For each immunohistochemical experiment, the brain sections were first of all dewaxed with xylene and dehydrated in 100% ethanol. The sections were then

incubated in H₂O₂ (1% in methanol), in order to block endogenous peroxidase activities, rinsed in ethanol and citrate buffer (10 Mm sodium citrate, pH 6) and finally placed in a microwave oven (650W, Whirlpool) for 5 2 × 5 min in the citrate solution. For the experiments in which the A-β protein was immunolabelled, the sections were subjected to an additional step, i.e. a 3 min incubation in 80% formic acid.

10 7.2 Thioflavin S

The sections were stained with 1% thioflavin S (Sigma, France) after having been incubated for 10 min in Mayer's haematoxylin solution (Sigma, France) in order to block nuclear fluorescence.

15 An FITC filter was then used to observe them in a microscope equipped with a fluorescence system (Axioscop Zeiss, France).

7.3. Congo Red

20 A polarized light system was used to analyze the sections which were stained with Congo red (Amyloid staining kit, Accustain, Sigma, France). Only the birefringent (to the rotation of the polarizer) red deposits were regarded as being Congo red-positive
25 deposits.

7.4. Immunoenzymic labelling

After having been incubated for 30 min in blocking buffer (10% normal goat serum (Chemicon) in PBS containing 0.1% triton (Sigma)), the dewaxed brain sections were incubated in the solution of the primary Ab (overnight at 4°C). After having been rinsed several times, the sections were brought into contact with the biotinylated secondary Ab (at ambient temperature for 2 h) and then into contact with the avidin-biotin peroxidase complex in accordance with the manufacturer's (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) instructions. 3-3'-Diaminobenzidine was used as the chromogen for the peroxidase enzyme.

For the preabsorption experiments, the anti-Bax Ab (antibody P19, Santa Cruz) was incubated with the synthetic Bax peptide (Control peptide P19, Santa Cruz) (tested concentrations of the peptide: 0.002, 0.02 and 0.2 mg/ml) for at least 12 h before being used in accordance with the previously described immunohistochemical protocol. The anti-cytochrome C Ab (7H8.2C12, Pharmingen) was incubated, using the same protocol, with exogenous purified cytochrome C from horse or rat heart (Sigma) (tested concentrations of the purified proteins: 0.01 and 0.1 mg/ml).

25 7.5. Quantifying the amyloid load

The deposition of A- β in A β -immunolabelled (biotinylated mouse anti-A β_{17-24} , monoclonal Ab 4G8,

Senetek) 25 µm hemibrain sections was quantified using diaminobenzidine as the chromogen and an image analysis system coupled to a color camera and a microscope (Q600 system, LEICA). Video images of each anatomical region 5 of interest were captured and a threshold was defined for automatically detecting the mean gray level corresponding to the immunolabelling of the A-β deposits (and capable of distinguishing the specific labelling from the background noise). The experimenter 10 checked each field manually in order to eliminate any artifact by manual means. In each mouse, the A-β load was measured at the bregma rostrocaudal level -3.4 of the mouse stereotactic atlas (Franklin and Paxinos). The load of A-β is defined as being the percentage of 15 the area of A-β immunolabelling with respect to the total area of the cerebral region analyzed, that is the hippocampus, the cortex and the remainder of the section (subcortical regions).

20 **7.6. Double immunoenzymic labelling**

The immunohistochemical double-labelling experiments were carried out by incubating the brain sections in accordance with a two-step protocol. In brief, the sections were immunolabelled in a first step 25 using a primary Ab (for example: anti-GFAP, anti-synaptophysin, anti-Bax or anti-cytochrome C), with this antibody being visualized by means of a brown

labelling obtained using diaminobenzidine (enzymic substrate for the horseradish peroxidase) in accordance with the previously described protocol. The same sections were then immunolabelled using the primary 5 anti-A β ₁₇₋₂₄ Ab (mouse monoclonal Ab 4G8, Senetek), with this antibody being visualized by means of a blue labelling obtained using the horseradish peroxidase substrate SG (SG peroxidase substrate kit, Vector).

10 7.7. Double immunofluorescent labelling

A double immunofluorescent labelling was performed in some experiments, in particular with the aim of showing whether Bax collocates with APP, PS1, cytochrome C or SMI in axonal structures associated 15 with the A- β deposits in transgenic mice carrying a double mutation (APP/PS1 mutant). In brief, the sections were first of all incubated in the anti-Bax Ab and then visualized either using a secondary biotinylated rabbit anti-IgG Ab which was conjugated to 20 CY3 (1:400, Chemicon) or using a secondary rabbit anti-IgG Ab and then a signal amplification kit (Streptavidin-peroxidase/tetramethyl-Rhodamine Tyramide complex) in accordance with the manufacturer's (New England Nuclear) instructions. For the second 25 immunofluorescent labelling, the sections were incubated consecutively with a primary anti-APP, anti-PS1, anti-cytochrome C or anti-SMI Ab and then

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with a secondary biotinylated mouse or rat anti-IgG Ab. The sections were finally visualized using a second signal amplification system (Streptavidin-peroxidase/ Fluorescein Tyramide complex) in accordance with the 5 manufacturer's (New England Nuclear) instructions. The sections were observed under the microscope using CY3 or rhodamine filters (excitation at 550 nm, emission at 570 nm) in the case of the Bax immunofluorescent labelling and a fluorescein filter (excitation at 10 495 nm, emission at 517 nm) in the case of the second immunofluorescent labelling. In the same way, in the case of the double immunofluorescent labelling experiments which were performed with the aim of showing whether cytochrome C collocates with the delta- 15 catenin protein, the sections were firstly incubated in the anti-cytochrome C Ab and then visualized using the Rhodamine fluorescence system. In a second step, these same sections were incubated in the anti-delta catenin Ab and visualized using the Fluorescein fluorescence 20 system as described above.

EXAMPLES

EXAMPLE 1: Analysis of the levels at which the 25 transgene is expressed

1.1 Comparison of the levels at which the transgene is expressed in different transgenic mice expressing mutated APP

Brain homogenates from different transgenic 5 mouse lines (different expression constructs) were analyzed for APP expression by means of Western blotting using a WO-2 monoclonal antibody. This antibody is specially indicated since it recognizes human APP and A- β but does not recognize the endogenous 10 mouse APP. Thus, no signal was detected in the lane corresponding to the nontransgenic mouse (Fig. 1, lane 3). Lanes 1 and 2 in Figure 1 correspond to brain extracts from transgenic mice which were previously shown to express high levels of the APP transgene 15 (Moechard et al. 1999a). The transgenic PDGF APP₆₉₅SDL mice in lane 7 were used to produce double transgenic mouse lines together with the mice carrying the PS1 M146L transgene. A moderate level of APP expression can be seen in the mice having the HMG promoter in the 20 construct (Czech et al. 1997). Note that, in the mice which are transgenic for the Swedish mutation form of APP, the beta-secretase fragment (12 KDa) is increased relative to the complete APP holoprotein.

25 1.2 Western blot analysis of the expression, and the processing, of the transgene in the brains of APP and Presenilin double transgenic mice

In order to find out whether the expression of each individual transgene was modified in the mice carrying the two transgenes (obtained by crossing), homogenates of brains from double transgenic mice of 5 different ages were analyzed for expression of APP and A- β by means of Western blotting using the WO-2 monoclonal antibody (described above). No difference in the intensity of the bands corresponding to the complete 100 kDa APP sequence and the 12 kDa 10 aminoterminal fragment is visible between the PDGF-APP₆₉₅SDL (monotransgenic) mice and the APP₆₉₅SDL × PS1M146L double-transgenic mice (Fig. 2A, compare also with lane 7, Fig. 1). In addition, the ages of the transgenic mice do not have any effect on 15 the expression of the APP transgene or on its metabolism at the level of the beta cleavage site. However, A- β accumulates strongly at 9 months, with this accumulation beginning at the age of 6 months. This increase in A- β correlates well with the beginning 20 of the formation of amyloid plaques in the brains of the transgenic mice (see below). This suggests that the increase in A- β which is detected by Western blotting may correspond to the A- β which has accumulated in the amyloid plaques.

25 Another Western blot analysis was performed on brain homogenates in order to determine whether the human PS1 protein is expressed in the double-transgenic mice and

in the nontransgenic (littermate) control mice. A monoclonal Ab was used which was directed against the aminoterminal part of PS1. This Ab is specific for human PS1; no signal for the endogenous PS1 protein is detected in the cerebral homogenate from the single-mutant APP mouse (Fig. 2B, lanes 1 and 2). The characteristic aminoterminal PS1 fragment is detected in the brain of the mouse expressing PS1M146L (Fig. 2B, lanes 3-7), with the appearance of the complete PS1M146L holoprotein (approx. 51 kDa), probably due to saturation of the presenilin processing, as has previously been described in transgenic rats and mice expressing high quantities of PS1 (Czech et al., 1998; Thinakaran et al., 1996). The expression and processing of human PS1 in the double-transgenic mouse do not vary with the age of the animal.

In conclusion, the levels at which the individual transgenes (APP or PS1) are expressed are similar in the monotransgenic and double-transgenic mice.

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EXAMPLE 2 Location and regional expression of the transgenes in single (PDGF APP₆₉₅SDL or HMG PS1M146L) or double (PDGF APP₆₉₅SDL x HMG PS1M146L) mice.

The immunohistochemical experiments on APP, 25 using the mouse monoclonal Ab (Ab 22C11) directed against the APP protein, demonstrated that single-transgenic (PDGF APP₆₉₅SDL) and double-transgenic

(PDGF APP₆₉₅SDL × HMG PS1M146L) mice express the APP protein more strongly than do nontransgenic control mice (Fig. 3). This result confirms that these two transgenic mouse lines express the human APP protein encoded by the APP₆₉₅SDL transgene. The human APP is located exclusively in the neurons. Its pattern of expression is similar in the two transgenic mouse lines and involves a large number of cerebral structures, with the highest level of expression being observed in the neuronal layers of the hippocampal formation (Fig. 3), followed by a similar level of expression in the cortical regions, such as the entorhinal cortex, and in the amygdala. The subcortical regions exhibit a lower level of expression of the transgene, and the human APP protein is not detected either in the glial cells or at the vascular level.

In the same way, the immunohistochemical experiments on PS1, using the rat monoclonal Ab directed against the human PS1 protein (Ab which specifically recognizes human PS1), demonstrated that the single-transgenic (HMG PS1M146L) and double-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L) mice express the human PS1 protein in the brain with specific location in the neurons (Fig. 3), although some immunolabelled glial cells are occasionally observed. The pattern of expression of the human PS1 protein is similar in the two transgenic mouse lines. Its level of

expression is elevated in the cortical structures such as the hippocampal formation (Fig. 3) and different regions of the cortex, and also in the subcortical regions. The protein is not expressed in the white substance. Both in the case of the human APP protein and in the case of the human PS1 protein, neither the pattern nor the level of expression changes with age (from 6 to 12 months).

10 EXAMPLE 3 Demonstration that the process of amyloid deposition is accelerated in the brains of double-transgenic mice (PDGF APP₆₉₅SDL × HMG PS1M146L) which are carrying both the mutant APP protein and the mutant PS1 protein.

15

We used both a variety of anti-A β Abs (see Materials and Methods) and conventional histological markers (Thioflavin S and Congo Red), which were known to stained A- β deposits in human tissues from patients suffering from AD, for showing whether the different transgenic mouse models which had been generated developed A- β deposits in the brain.

Using the set of A- β deposit markers, we showed that none of the single-transgenic mice, i.e. PDGF APP₆₉₅SDL or HMG PS1M146L, exhibited any amyloid deposition in the brain at the ages of 3, 6, 9, 12 and 15 months. On the other hand, the double-transgenic

PDG_F APP₆₉₅SDL × HMG PS1M146L mice exhibited A-β deposits from the age of 6 months onwards. The various anti-Aβ Abs employed all demonstrate the existence of this process of amyloid protein deposition (Figs. 4 and 5). These A-β deposits are also stained with Thioflavin S and Congo Red, thereby confirming their fibrillary conformation (Fig. 6).

EXAMPLE 4: Progression and regional pattern of amyloid deposition in the double-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L) mice

With the aim of assessing the progression and pattern of regional distribution of the amyloid deposition resulting from transgene expression in the double-transgenic mice (PDGF APP₆₉₅SDL × HMG PS1M146L), the number of A-β deposits in 6 µm-thick hemibrain sections at different ages, and the amyloid load in 25 µm-thick sections at 12 months of age, were quantified using the anti-Aβ Ab 4G8. We focused on the cortex and hippocampus for quantifying the amyloid load, since these two cerebral structures are involved in an early and predominant manner in the neuropathology of the transgenic mice and patients suffering from AD.

The number of A-β deposits is higher in the 12-month-old mice than in the mice aged 6 and 9 months, as Figures 7 and 8 demonstrate qualitatively and

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quantitatively, respectively. At 6 and 9 months, the A- β deposits are located in a limited cerebral region corresponding to the subiculum and to the dorsal part of the hippocampal region CA1 (Fig. 7). At 12 months, 5 on the other hand, the more numerous A- β deposits are located over the whole of the hippocampal and cortical regions along the whole length of the rostrocaudal axis of the brain (Fig. 7 and Fig. 9). In 12-month-old mice, the A- β deposits are only occasionally present in some 10 subcortical structures (for example the internal capsule, the dorsolateral thalamus and the basal ganglia). They are not present in either the cerebellum or in the spinal cord. Quantitative analysis of the amyloid load in 12-month-old mice (Fig. 10) 15 demonstrates that the percentages of the A β immunolabelling area with respect to the total area of the analyzed region reach values of 3.1; of 0.8 to 1.1, and of < 0.5 for the hippocampus, the cortex and the remainder of the hemibrain, respectively. The amyloid 20 load reaches more than 9% and 5% in the richest areas of the hippocampus and cortex, respectively. These amyloid loads are of a similar order of magnitude to those previously described in the human AD brain (6-12%) (Hyman et al., 1993).

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EXAMPLE 5: Axonal structures in the double-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L) mouse

In all the transgenic mice which develop A- β deposits, the deposits of large and medium size contain numerous axonal structures, as is demonstrated, on the one hand, by the pictures of single immunolabellings using different markers for dystrophic axons, such as APP (Fig. 11), PS1 (Fig. 12) and delta-catenin (Fig. 13), and, on the other hand, by the pictures of double immunolabellings, which visualize the A- β depositions, using the anti-A β Ab 4G8, and the axonal structures, using the anti-synaptophysin Ab or the anti-SMI Ab, in one and the same brain section (Fig. 14 and Fig. 15, respectively). As Figures 14 and 15 show, synaptophysin-immunoreactive and SMI-immunoreactive axonal structures are present in the brains of the double-transgenic mice and are intimately associated with the A- β deposits. Furthermore, our results demonstrate that the axonal structures are very similar to those described in the human AD brain.

In order to show whether neurofibrillary degenerations are also present in the brains of the double-transgenic mice, a double immunolabelling was performed for the purpose of visualizing the A- β deposits, using the anti-A β Ab 4G8, and the neurofibrillary degenerations, using the anti-Tau-I Ab, in one and the same brain section. As Figure 16 shows, the anti-Tau-I Ab labels axonal structures such as the

axons which carpet the A- β deposits and the abnormal cell bodies. While these two types of immunolabelled structure are present in the cerebral regions which are rich in A- β deposits (hippocampal formation and cortex), they are absent from the subcortical regions which are poor in, or lack, A- β deposits.

Taken as a whole, these results clearly demonstrate that, in addition to the phenomenon of amyloid deposition, degenerative processes take place within the cognitive structures of the double-transgenic mice in a manner similar to those in the AD brain.

EXAMPLE 6: Presence of activated astrocytes within or around the amyloid deposits in the double-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L) mouse

Inasmuch as the A- β deposits in the AD cerebral tissue are associated with glial cells (the inflammatory cells of the central nervous system), an investigation was carried out to determine whether a glial reaction takes place in the neuropil of our transgenic mice which develop A- β deposits. No GFAP-immunoreactive reactive astrocyte was observed in the brains either of the nontransgenic control mice or of the transgenic mice which did not develop A- β deposits (for example the single-transgenic

PDGF APP₆₉₅SDL or HMG PS1M146L mice). On the other hand, all the double-transgenic mice, having A- β deposits in their brains, possess GFAP-immunoreactive reactive astrocytes which are intimately associated with the A- β 5 deposits (Fig. 17).

**EXAMPLE 7: Demonstration of mitochondrial dysfunction
in the double-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L)
mouse**

10

A single immunolabelling was carried out in order to visualize the Bax protein on its own, and a double immunolabelling was carried out in order to visualize the Bax protein and the A- β deposits in one 15 and the same brain section; this was done with the aim of establishing a relationship between Bax expression and the amyloid plaques.

The results of the single labelling showed that the Bax protein is expressed in the neurons of a 20 large number of cerebral structures, such as the neuronal layers of the hippocampal formation and all the cortical regions (Fig. 18A to H), both in the non-transgenic control mice and in the transgenic mice.

However, only the double-transgenic 25 (PDGF APP₆₉₅SDL × HMG PS1M146L) mice, and not the single-transgenic (PDGF APP₆₉₅SDL × HMG PS1M146L) mice, or the nontransgenic control mice, exhibit Bax

immunoreactivity in cellular structures such as dystrophic axons (Fig. 18A to H), neuronal cell bodies resembling early neurofibrillary degenerations, and, occasionally, activated glial cells (see below).

5 Interestingly, these abnormal Bax-immunoreactive cell structures are located exclusively in cerebral regions which are rich in A- β deposits, such as the dentate gyrus, hippocampal regions CA1 and CA3, the subiculum and the cingulate and entorhinal cortices.

10 The double immunolabelling with the anti-Bax and anti-A β Abs subsequently confirmed that the Bax immunoreactivity in the axonal structures and the activated glial cells is intimately associated with the A- β deposits in the double-transgenic animals

15 (Figs. 18I to L). These Bax-immunoreactive cellular structures were never detected in the brains of the non-transgenic or single-transgenic animals. The Bax immunolabelling in these studies is specific, as the preabsorption experiments using a blocking peptide
20 demonstrated.

The results from the single immunolabelling of cytochrome C showed that the cytochrome C protein is expressed in the neuronal cell bodies in the 4 groups of animals examined (nontransgenic controls, and
25 single- and double-transgenic animals - Table I). The pattern of the distribution of the cytochrome C-immunoreactive neurons is similar to that described for

Bax. Interestingly, cytochrome C is shown, as in the case of Bax, to be expressed in cell structures such as axons and abnormal neuronal cell bodies (Fig. 20) only in the double-transgenic mouse. These cytochrome C-immunoreactive cell structures are never observed in the single-transgenic or nontransgenic control mice (Fig. 20). In the double-transgenic mice, the cytochrome C-immunoreactive axonal structures, like the Bax-immunoreactive axonal structures, are located in the cerebral structures where the A- β deposits are found, i.e. chiefly the dentate gyrus, hippocampal regions CA1 and CA3 and the cingulate cortices (Fig. 20). The double immunolabelling of the cytochrome C and the A- β deposits shows that, in these double-transgenic animals, the cytochrome C-immunoreactive axonal structures are intimately associated with the amyloid plaques (Fig. 20). The cytochrome C immunolabelling is specific, just like the Bax immunolabelling, as the preabsorption experiments using purified cytochrome C proteins demonstrated.

In parallel with the experiments performed on the transgenic mice, the Bax/A β and cytochrome C/A β double immunolabellings were carried out on postmortem human brain tissue from a control individual and from an AD patient. The results obtained (Fig. 19 and Fig. 21) show that, in the human AD brain, the Bax-immunoreactive or cytochrome C-immunoreactive

axonal structures such as dystrophic axons and early neurofibrillary neurodegenerations are present within or around the amyloid plaques. The Bax-immunoreactive and cytochrome C-immunoreactive abnormal neuronal 5 structures were not observed in the control human tissue.

In order to confirm the presence of the Bax and cytochrome C proteins in abnormal axonal structures in the brains of double-transgenic mice, double 10 immunofluorescent labellings were also performed which demonstrated that Bax was colocated with APP, PS1 and cytochrome C in the same axonal structures which carpet the A- β deposits (Figs. 22 and 23). Finally, it was shown that cytochrome C is colocated with delta-catenin 15 in these same axonal structures (Fig. 22).

The fact that Bax and cytochrome C are overexpressed in axonal structures such as axons and abnormal neuronal cell bodies both in the human AD brain and in the brains of the double-transgenic mice 20 demonstrates the involvement of these proteins in regulating early events associated with neuronal death in the AD patient and in this transgenic mouse model.

These findings in the mouse are the first to show that the expression of two different mitochondrial 25 proteins, i.e. Bax and cytochrome C, which are known to induce cell death in culture (see introduction), is altered in the brains of transgenic mice which develop

A- β deposits. This modified expression of Bax and cytochrome C consists in these two mitochondrial markers accumulating in abnormal neuronal structures which are intimately associated with the amyloid plaques.

An important point is that the Bax-immunoreactive and cytochrome C-immunoreactive axonal structures in the double-transgenic mouse resemble those observed in the human AD brain, indicating that the same type of mitochondrial dysfunction is found in the human AD patient.

Table I

$\text{A}\beta$ deposition and markers of apoptosis mediated by mitochondrial dysfunction in axonal

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CLAIMS

1. Non-human transgenic animal model of Alzheimer's disease which exhibits both amyloid plaques
5 and mitochondrial dysfunction.

2. Animal model according to Claim 1, characterized in that it coexpresses the β -amyloid peptide precursor (APP) and a presenilin, preferably presenilin 1 (PS1).

10 3. Animal model according to Claim 2, characterized in that it coexpresses mutated forms of APP and/or PS1.

4. Animal model according to Claim 3, characterized in that the mutation in the APP gene is
15 selected from the "Swedish", "London" and "Dutch" mutations, taken on their own or in combination.

5. Animal model according to Claim 3, characterized in that the mutation in the PS1 gene is selected from the M146L, A246E, C410Y, H163R, L286V and
20 L235P mutations, taken on their own or in combination.

6. Animal model according to Claim 5, characterized in that it is the M146L mutation.

7. Animal model according to Claim 1, characterized in that the mitochondrial dysfunction is
25 an alteration, a modification, an overexpression or an inhibition of the expression of the mitochondrial proteins.

8. Animal model according to Claim 7,
characterized in that the proteins are
intramitochondrial proteins.

9. Model according to Claim 8,
5 characterized in that the proteins are the BAX and/or
cytochrome C proteins.

10. Use of the animal model as described in
accordance with Claims 1 to 9 for identifying compounds
which can be used for treating neurodegenerative
10 diseases, preferably Alzheimer's disease.

11. Cell which is extracted from an animal
model as described in accordance with Claims 1 to 9.

12. Use of a cell as described in accordance
with Claim 11 for identifying compounds which can be
15 used for treating neurodegenerative diseases,
preferably Alzheimer's disease.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(43) International publication date
29 March 2001 (29.03.2001)

PCT

(10) International publication number
WO 01/20977 A1

(51) International patent classification ⁷ :	A01K 67/027, C07K 14/47, A61K 49/00, C12N 5/10, G01N 33/50	(74) Representative: BOUVET, Philippe; Aventis Pharma S.A., Direction Brevets, 20, avenue Raymond Aron, F-92165 Antony Cedex (FR).
(21) International application number:	PCT/FR00/02540	(81) Designated states (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
(22) International filing date:	14 September 2000 (14.09.2000)	(84) Designated states (regional): ARIPO Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(25) Language of filing:	French	
(26) Language of publication:	French	
(30) Data relating to the priority:	99/11,678 17 September 1999 (17.09.1999). 60/161,183 22 October 1999 (22.10.1999)	FR US
(71) Applicant (for all designated States except US):	AVENTIS PHARMA S.A. [FR/FR]; 20 Avenue Raymond Aron, F-92160 Antony (FR).	
(72) Inventors; and		Published:
(75) Inventors/Applicants (US only): MOUSSAOUI-MRABET, Saliha [MA/FR]; 166 Avenue Maréchal de Lattre de Tassigny, F-94120 Fontenay sous Bois (FR). BLANCHARD-BREGEON, Véronique [FR/FR]; 73, rue Bobillot, F-75013 Paris (FR). IMPERATO, Assunta [IT/FR]; 195 bis rue Raymond Losserand, F-75014 Paris (FR). BONICI, Bruno [FR/FR]; 1 rue des Charmes, F-91230 Montgeron (FR). TREMP, Gunter [DE/FR]; 6 résidence du Parc d'Ardenay, F-91120 Palaiseau (FR). CZECH, Christian [FR/FR]; 4 Cité de l'Alma, F-75007 Paris (FR).	<i>With the International Search Report.</i> <i>Before expiry of the period provided for amending the claims, will be republished if such amendments are received.</i>	

As printed

(54) Title: NOVEL ANIMAL MODEL OF ALZHEIMER DISEASE WITH AMYLOID PLAQUES AND MITOCHONDRIAL DYSFUNCTIONS

(54) Titre: NOUVEAU MODELE ANIMAL DE LA MALADIE D'ALZHEIMER PRÉSENTANT À LA FOIS DES PLAQUES AMYLOÏDES ET DES DYSFONCTIONNEMENTS MITOCHONDRIAUX

(57) Abstract: The invention concerns the field of transgenic animal models and more particularly, animal models of Alzheimer disease. The invention relates to a novel animal model of Alzheimer disease having both amyloid plaques and mitochondrial dysfunctions.

(57) Abrégé: La présente invention concerne le domaine des modèles animaux transgéniques et plus particulièrement, les modèles animaux de la maladie d'Alzheimer. L'invention se rapporte à un nouveau modèle animal de la maladie d'Alzheimer présentant à la fois des plaques amyloïdes et des dysfonctionnements mitochondriaux.

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PCT/FR00/02540

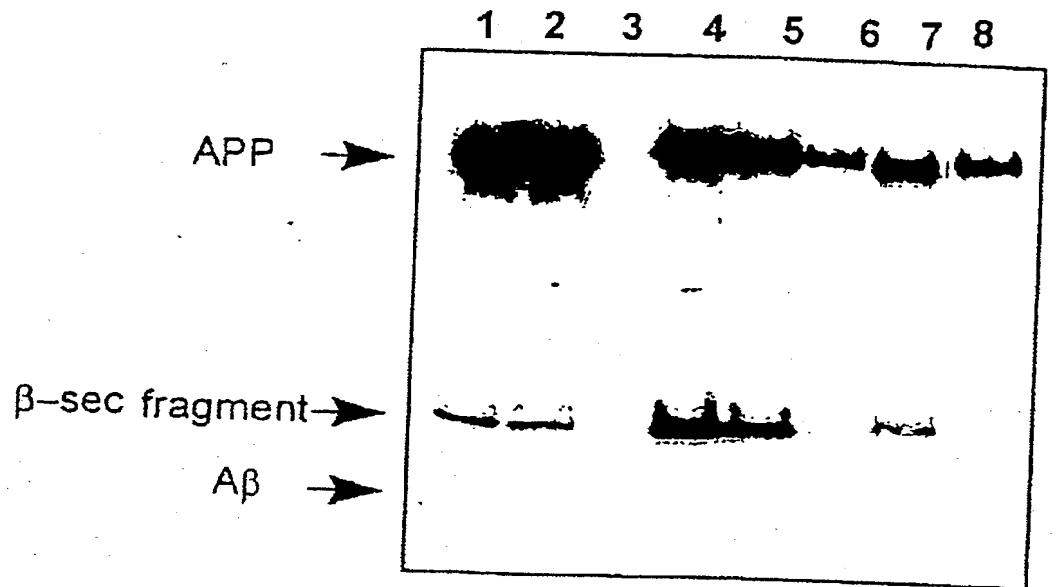


Figure 1

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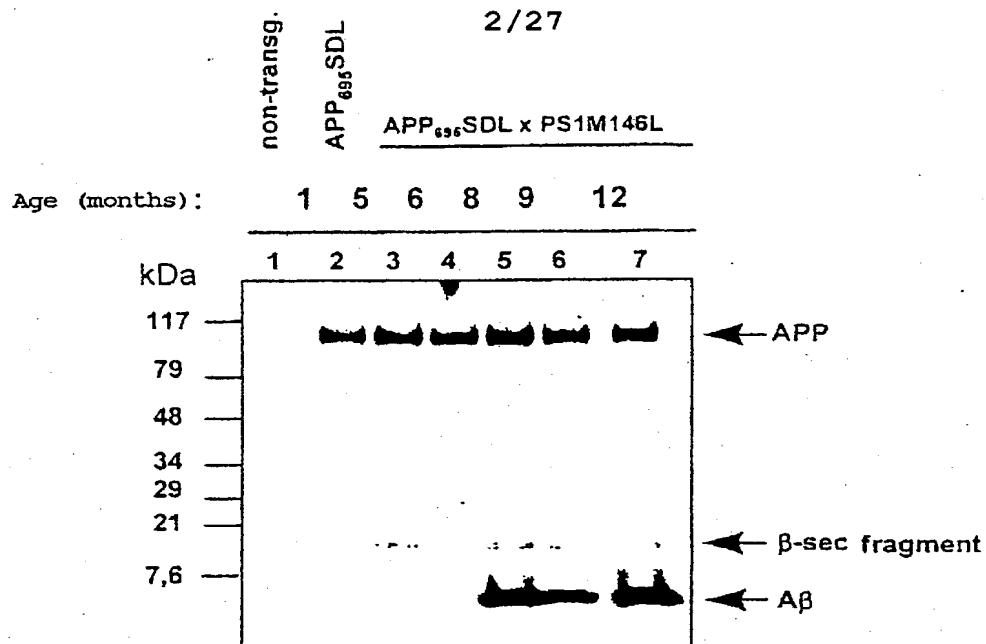


Figure 2A

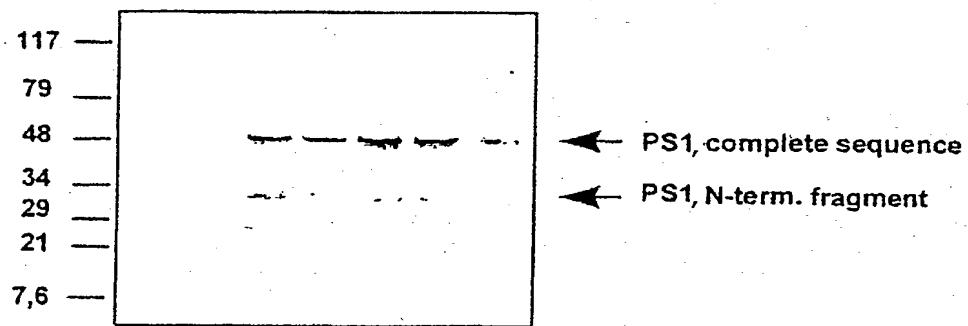


Figure 2B

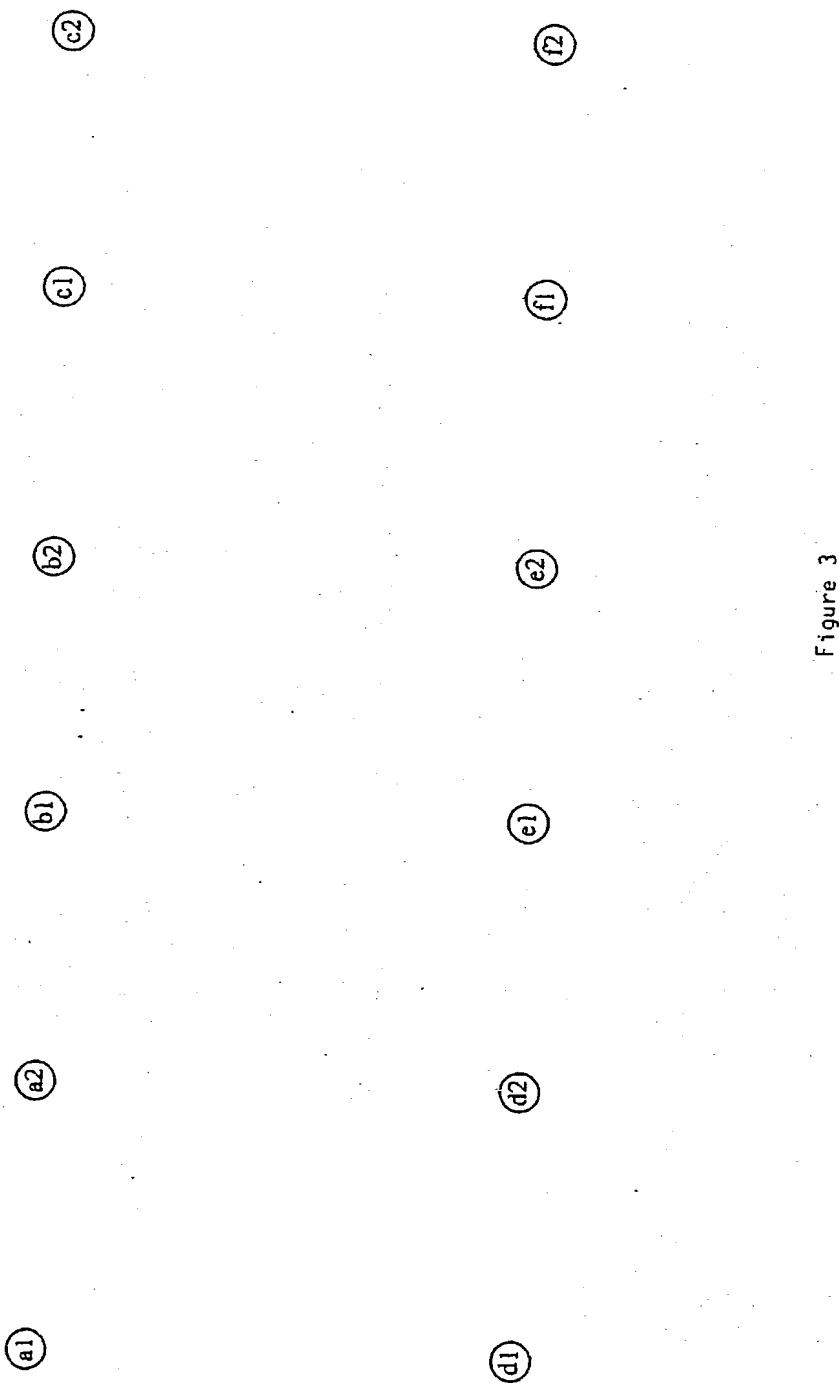


Figure 3

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Figure 4A
Figure 4B
Figure 4C

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PCT/FR00/02540

Figure 5C

Figure 5B

Figure 5A

Figure 5E

Figure 5D

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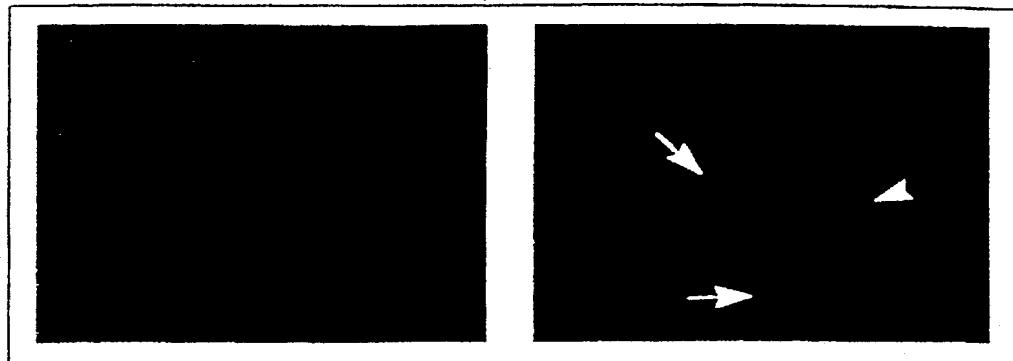


Figure 6A

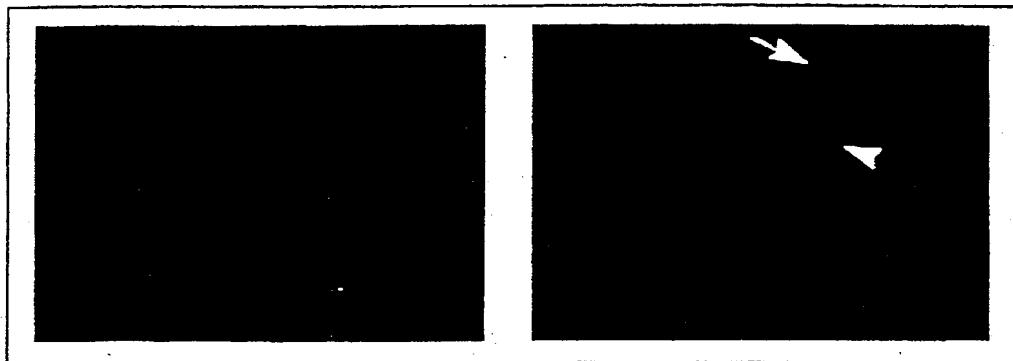


Figure 6B

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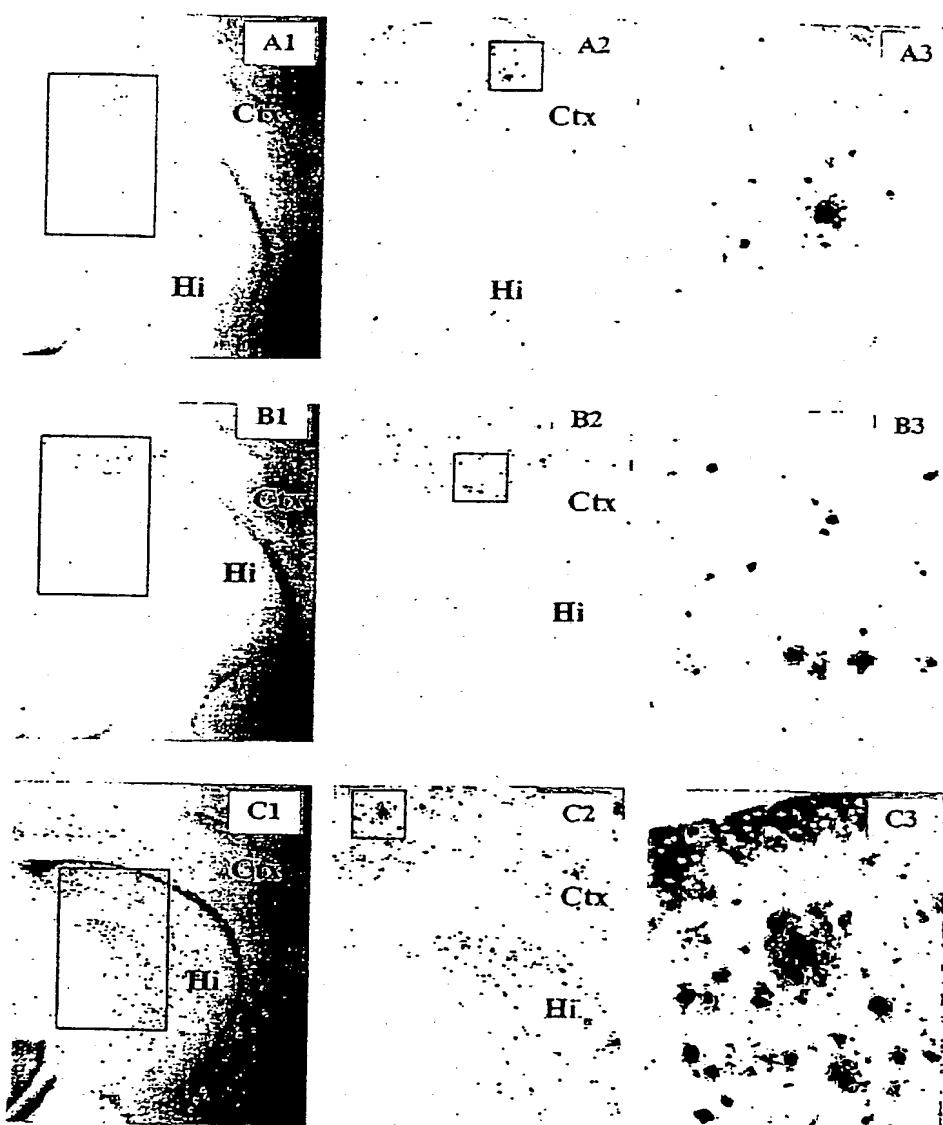


Figure 7

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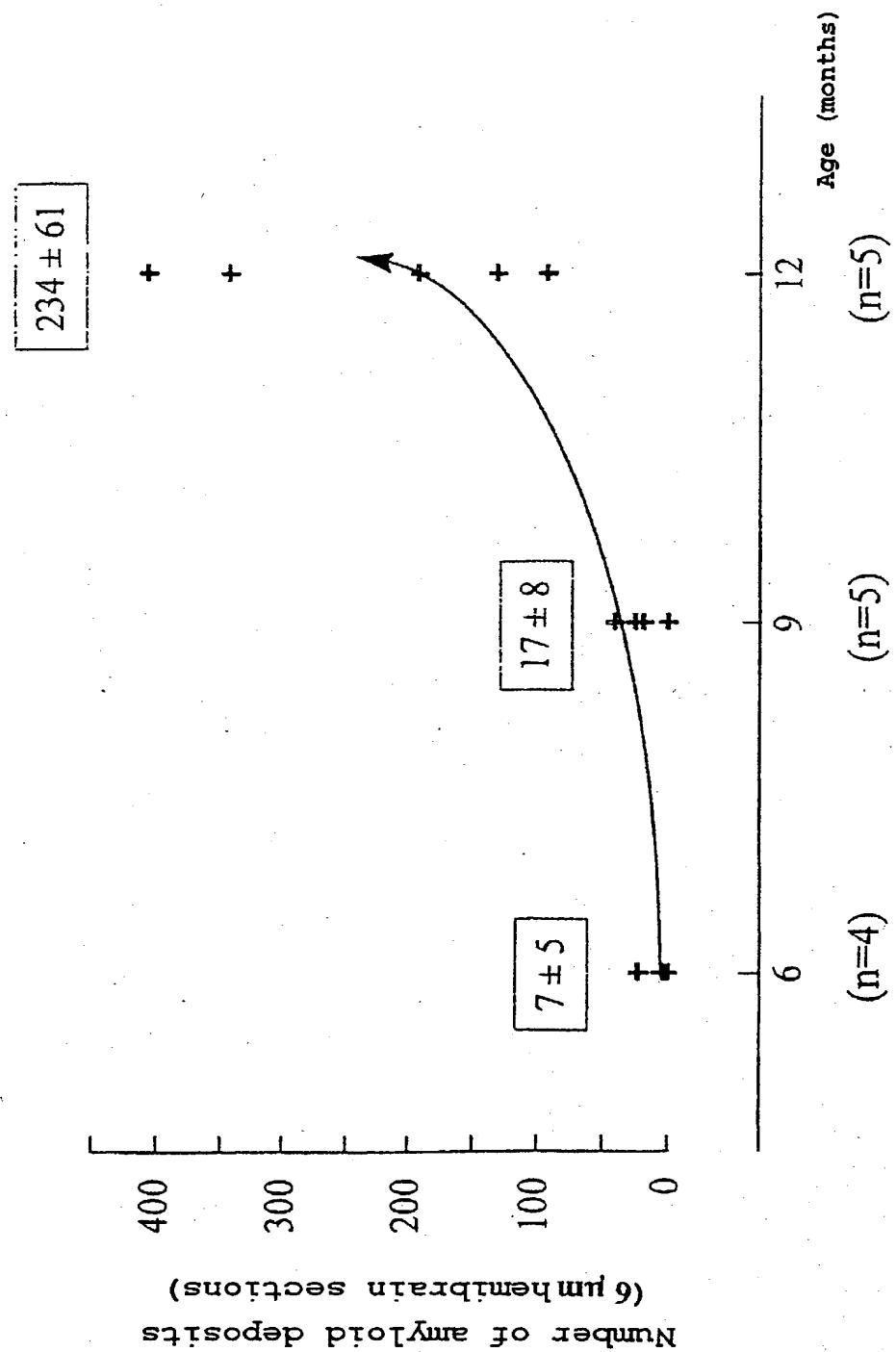


Figure 8

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Figure 9

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PCT/FR00/02540

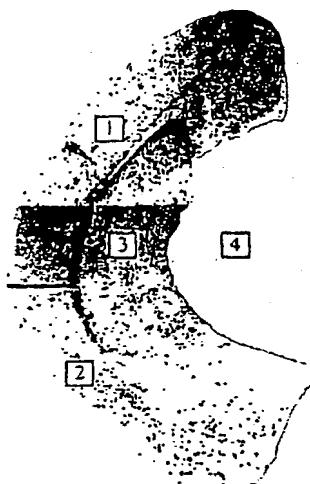


Figure 10A

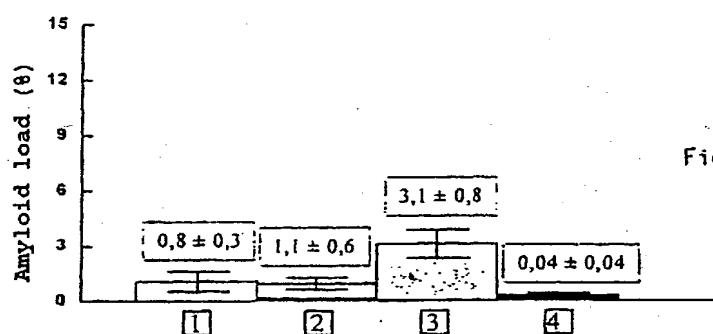


Figure 10B

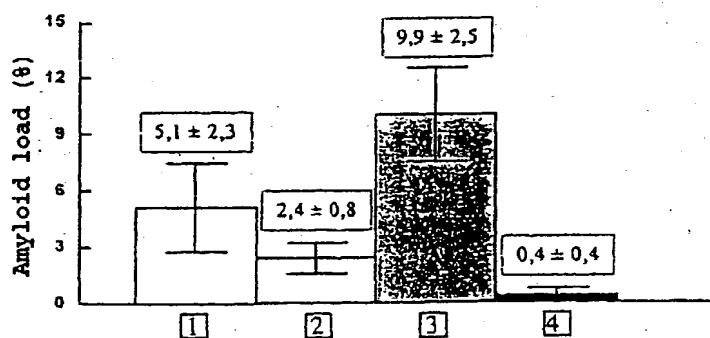


Figure 10C

Figure 10

4 0 0 0 0 0 4 2 0 - 1 0 7 0 8 8 1 3 8

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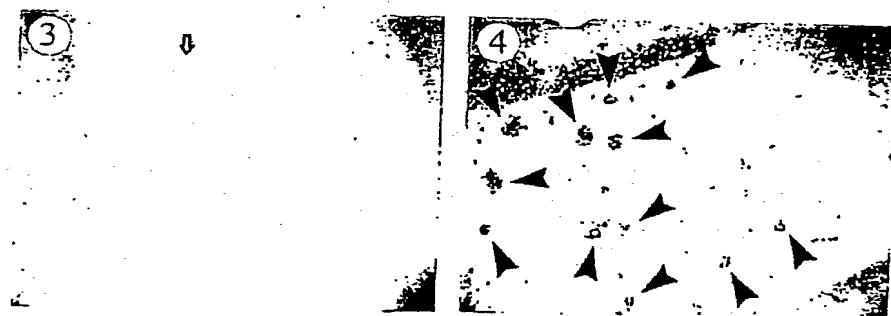


Figure 11A

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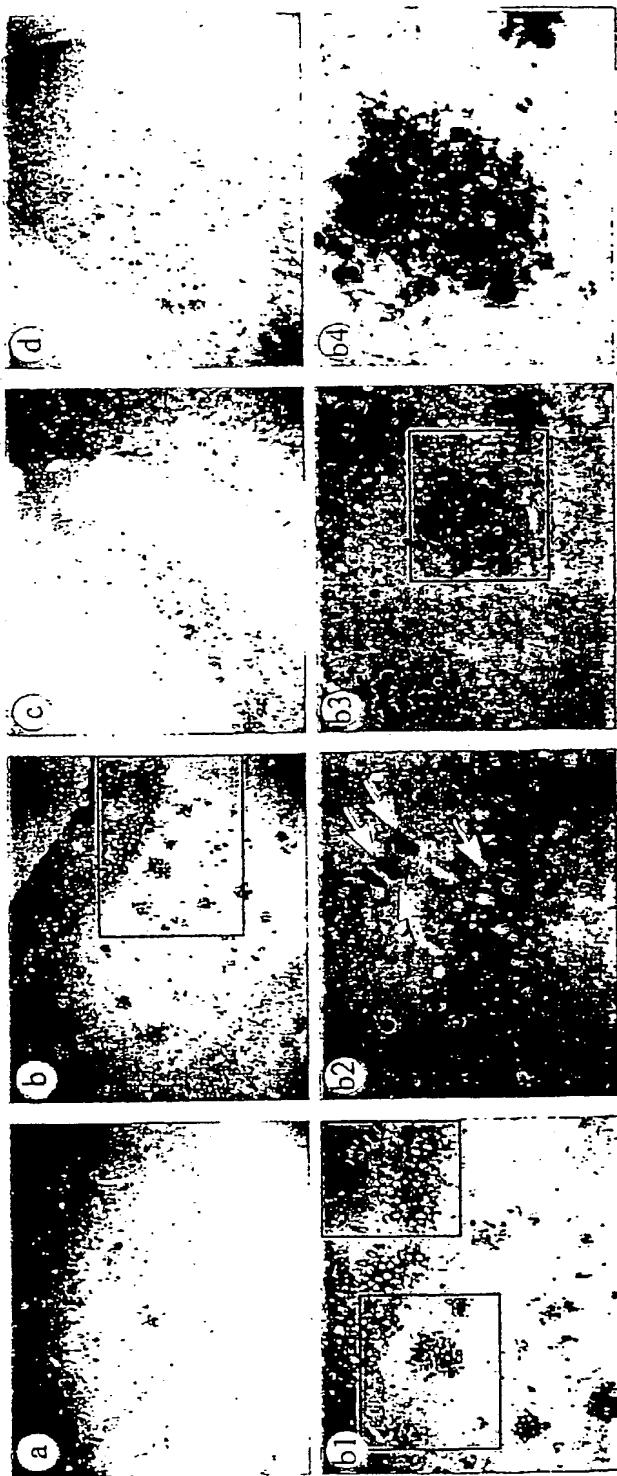


Figure 11B

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(1)

(2)

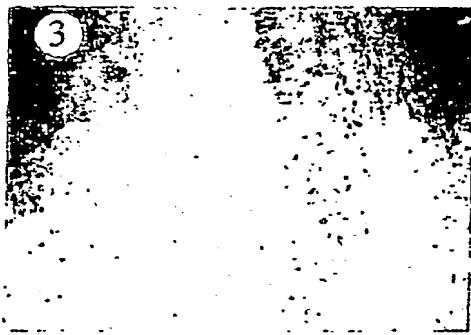


Figure 12A

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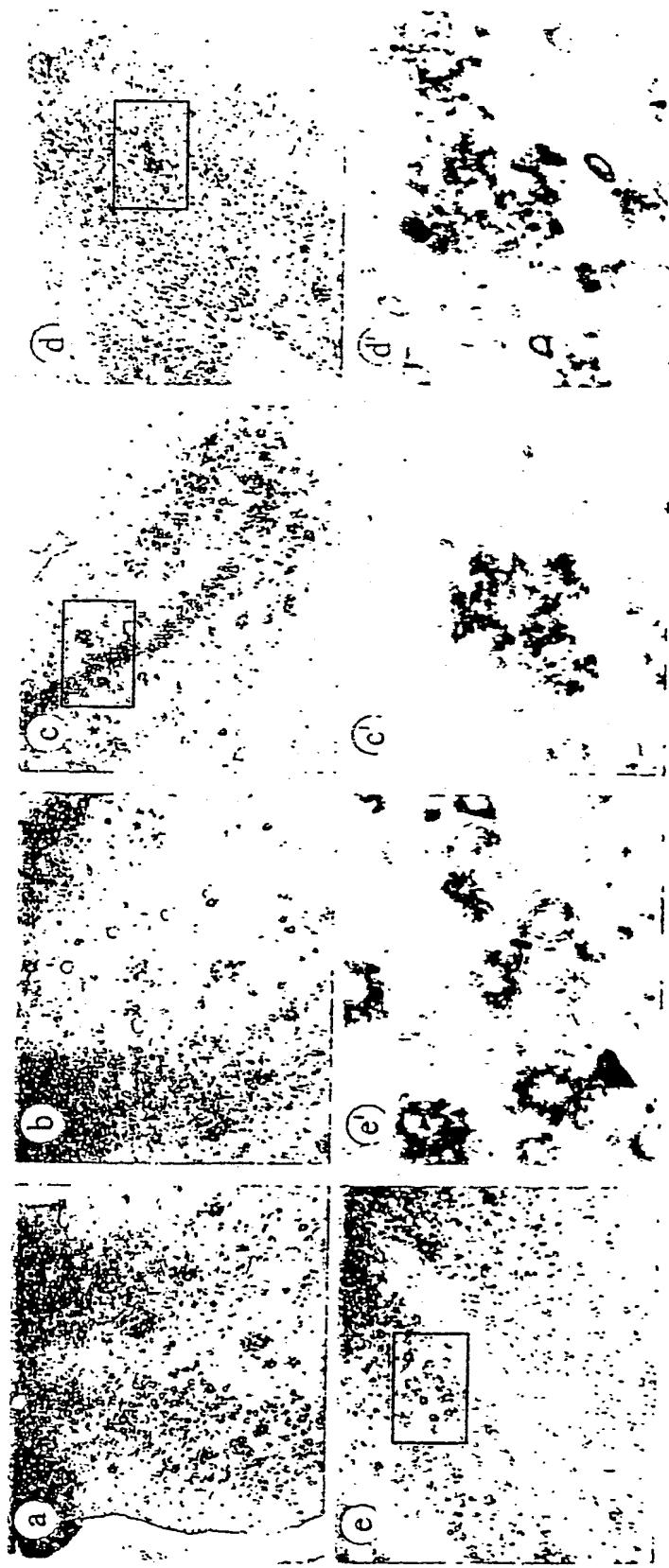


Figure 12B

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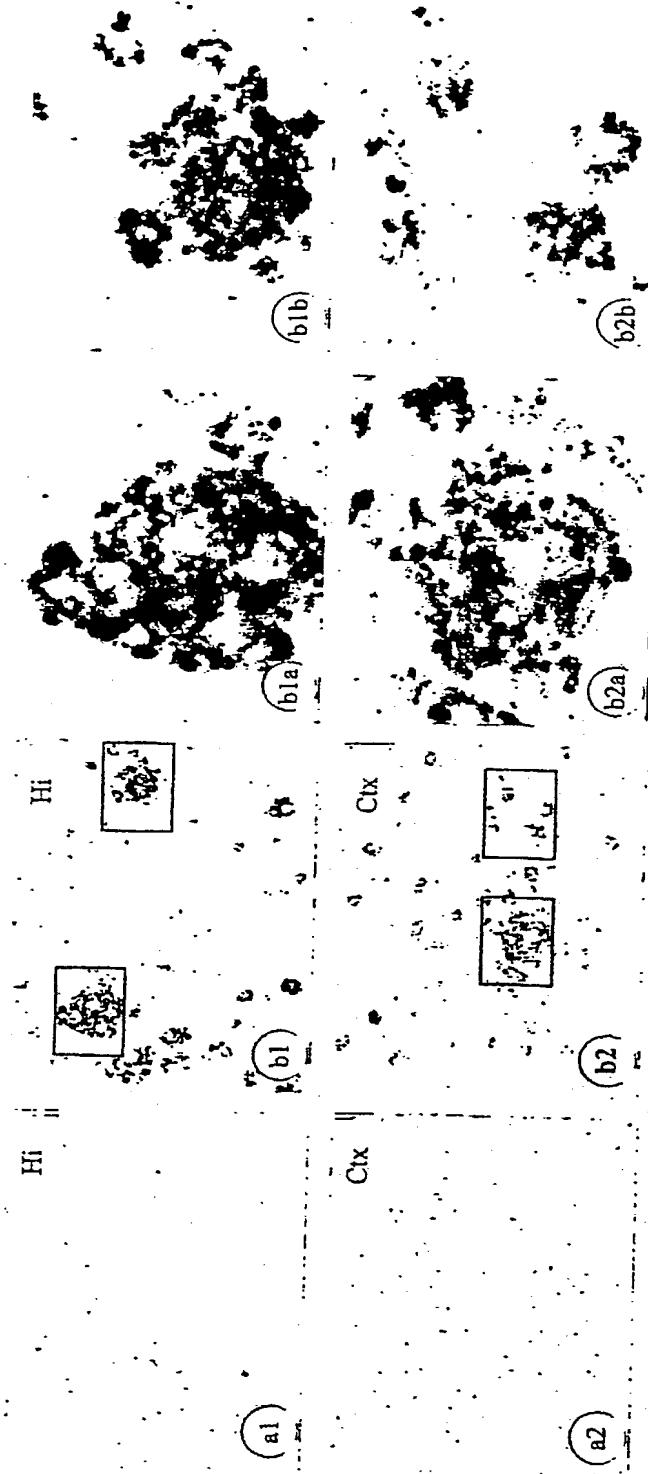


Figure 13

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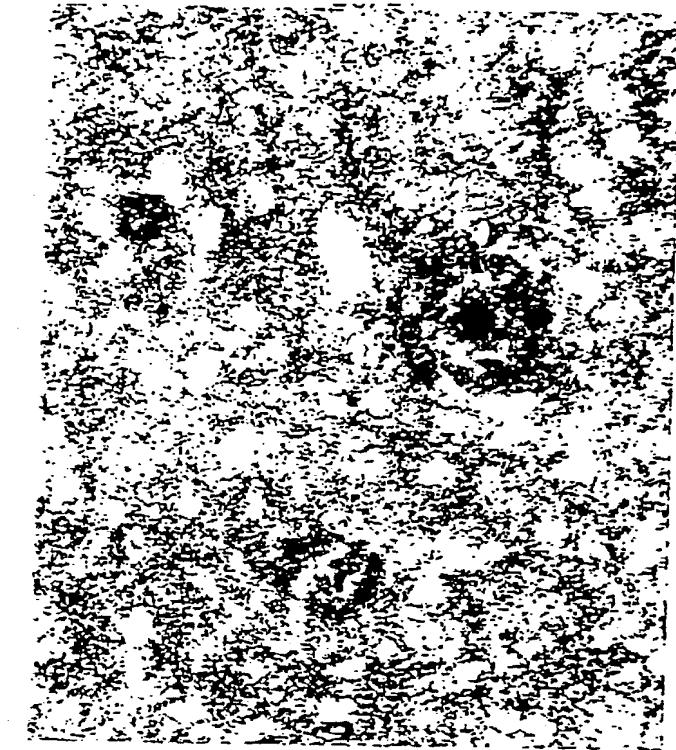


Figure 14B



Figure 14A

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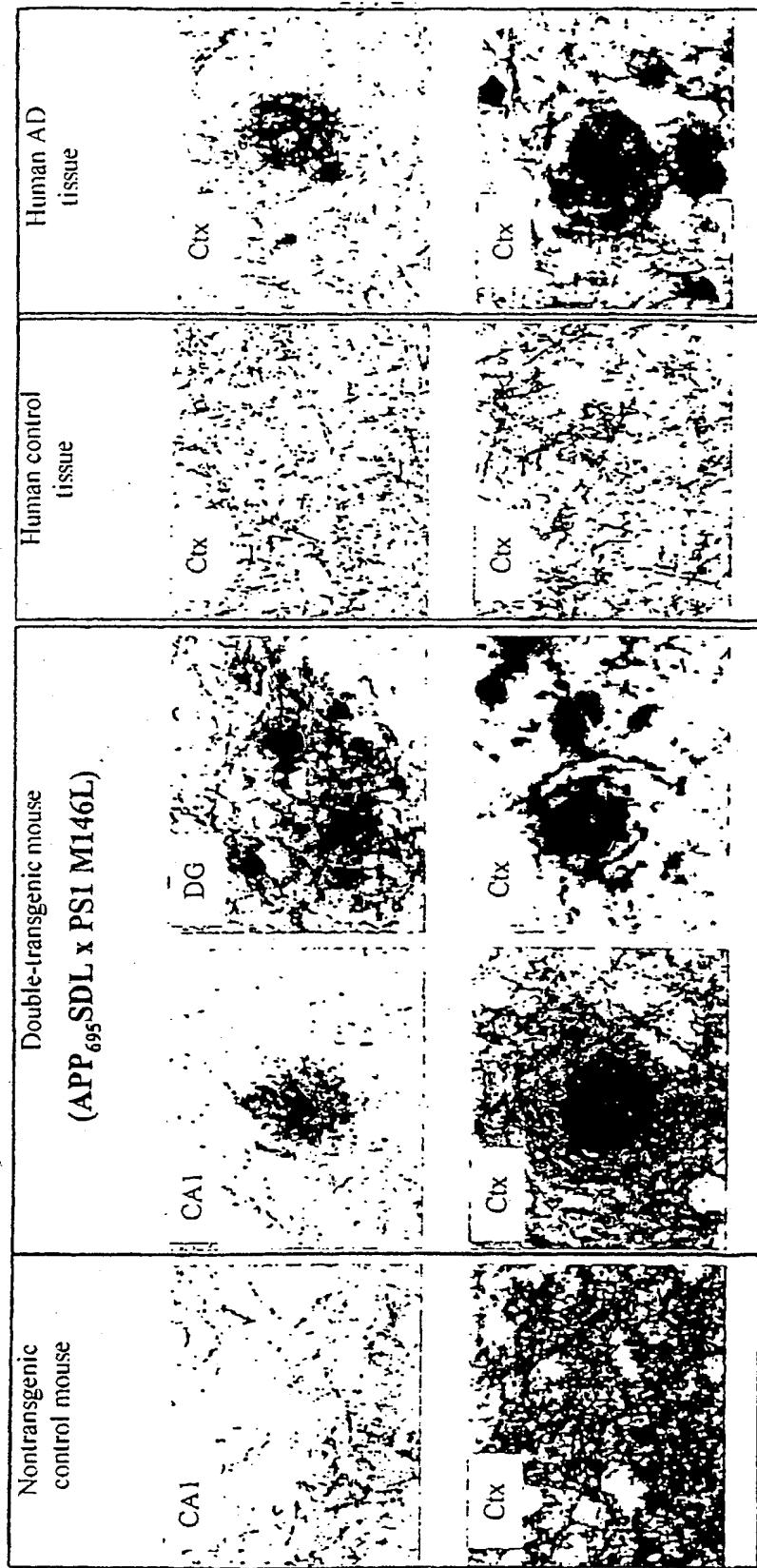


Figure 15

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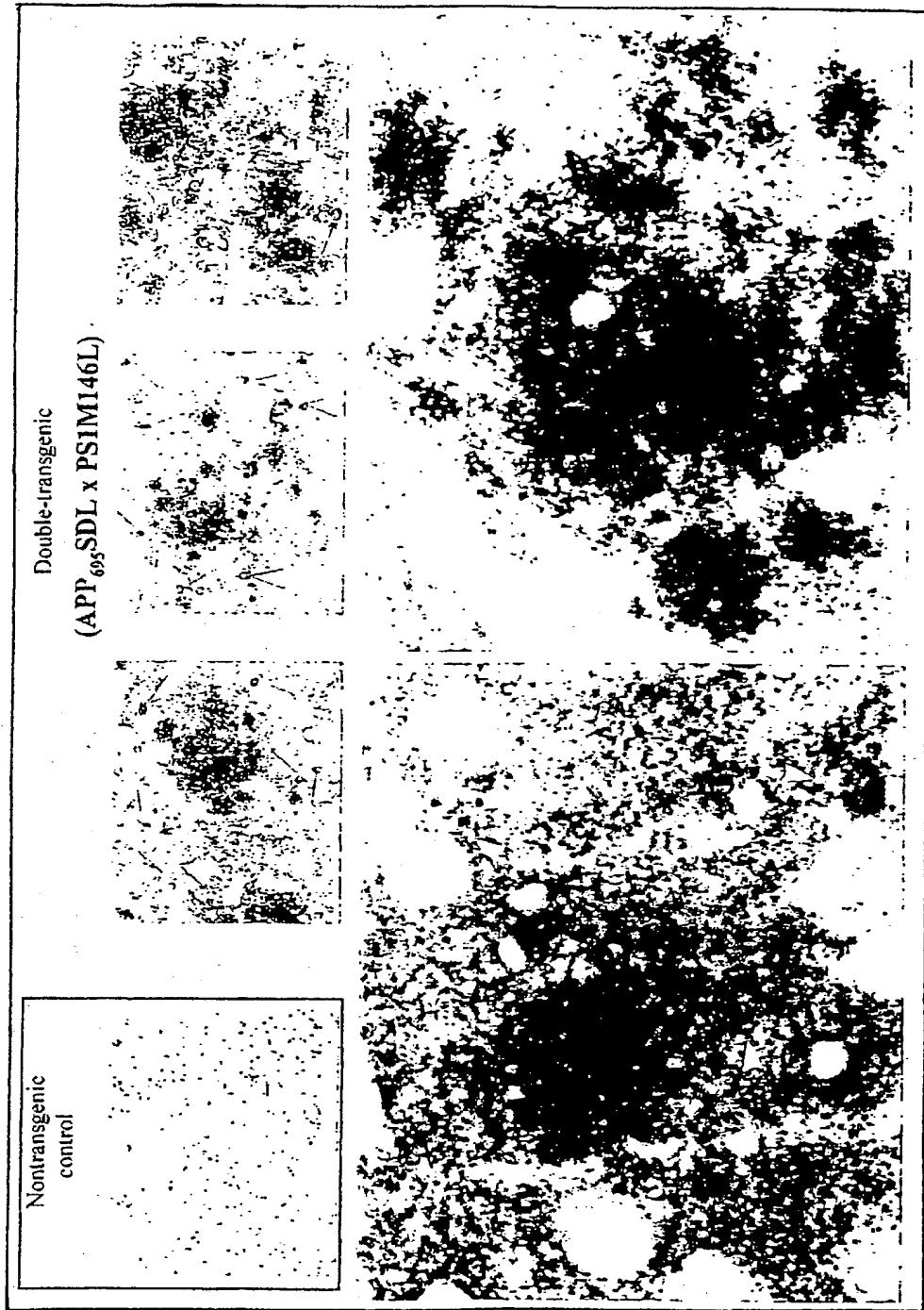


Figure 16

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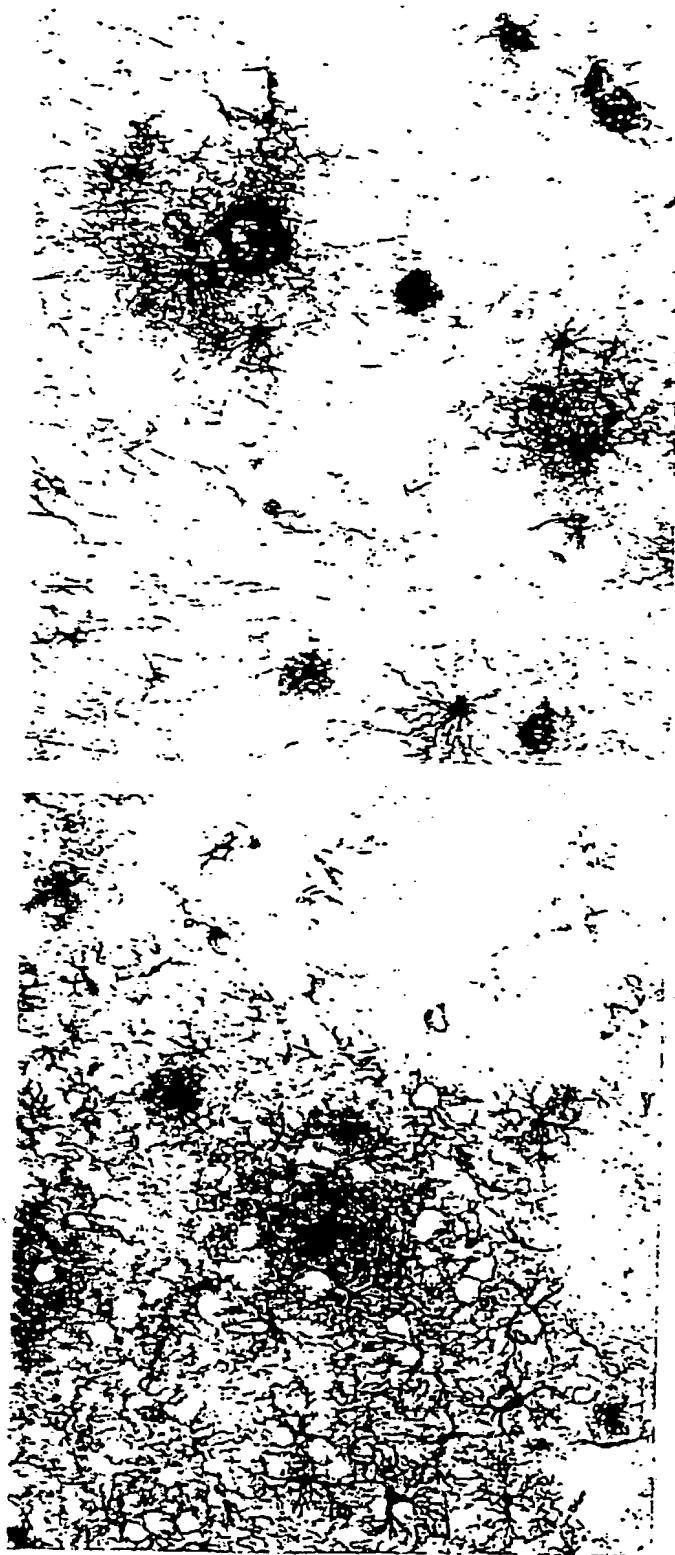


Figure 17B

Figure 17A

1.00000138 - 10/088138

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Figure 18A

Figure 18E

Figure 18B

Figure 18F

Figure 18C

Figure 18G

Figure 18D

Figure 18H



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Figure 18K



Figure 18I

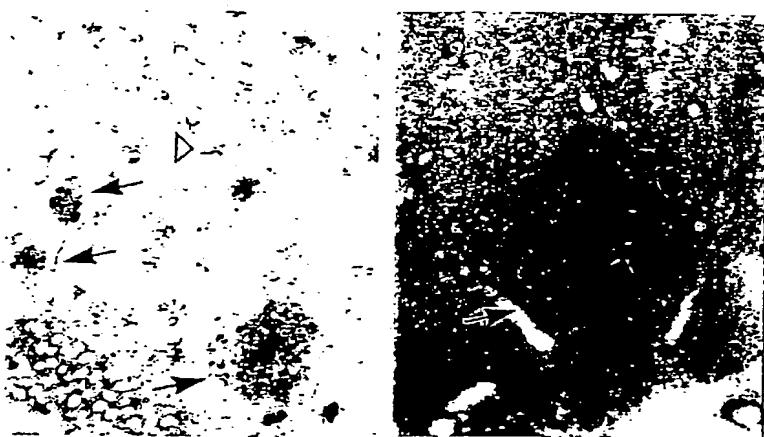


Figure 18L

Figure 18J

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Figure 19A

Figure 19C



Figure 19B

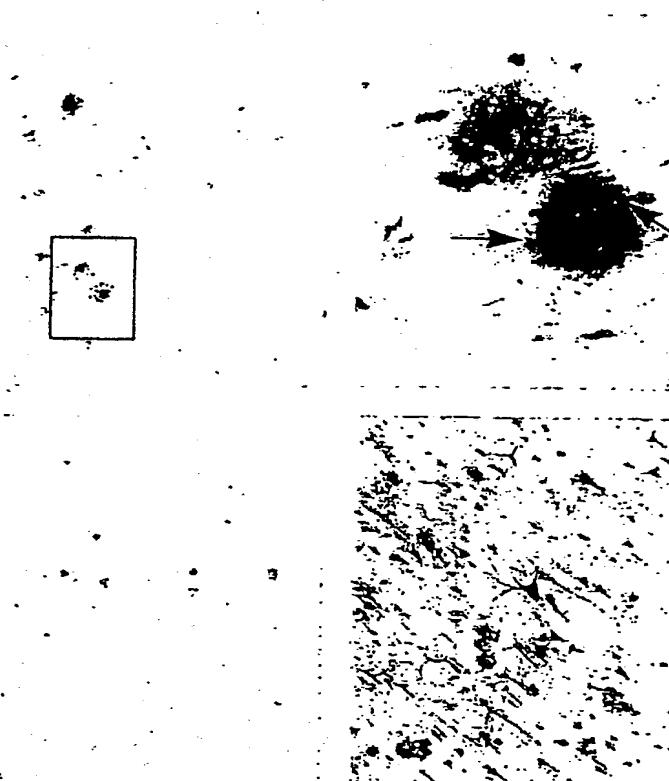


Figure 19D

Figure 19E

Figure 19F

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Figure 20A



Figure 20B

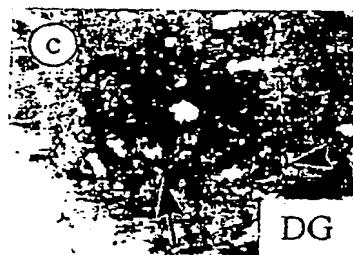


Figure 20C



Figure 20D



Figure 20E

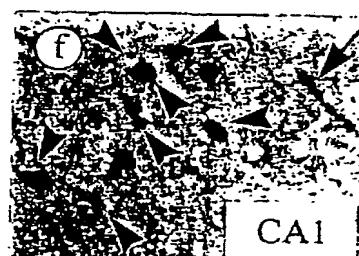


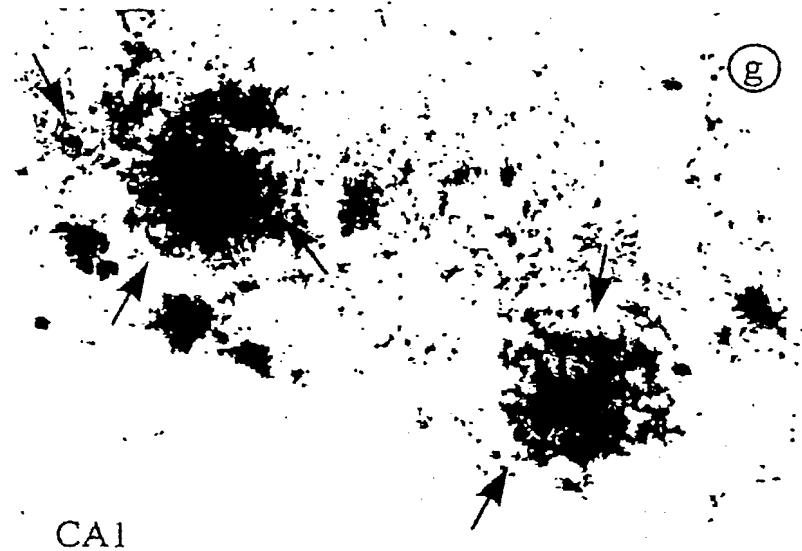
Figure 20F

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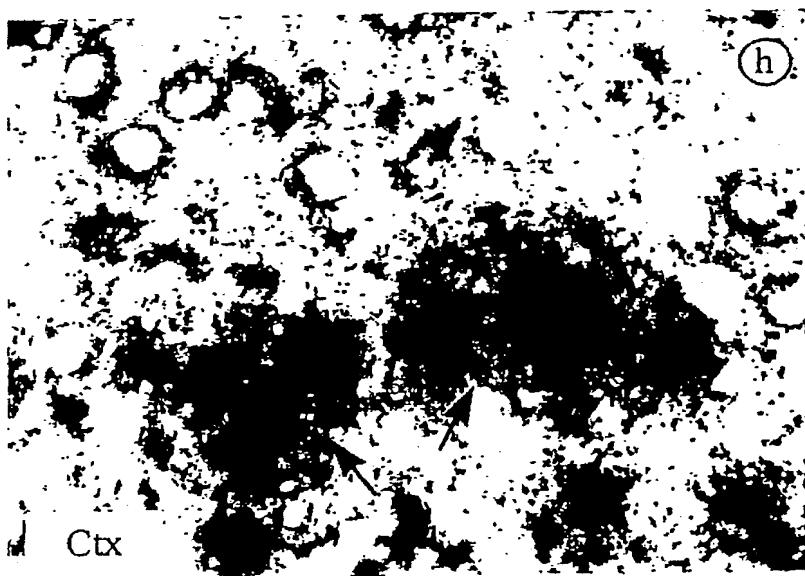
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CA1

Figure 20G



Ctx

Figure 20H

Figure 21A

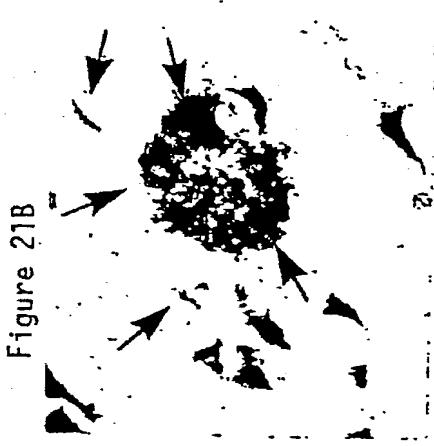
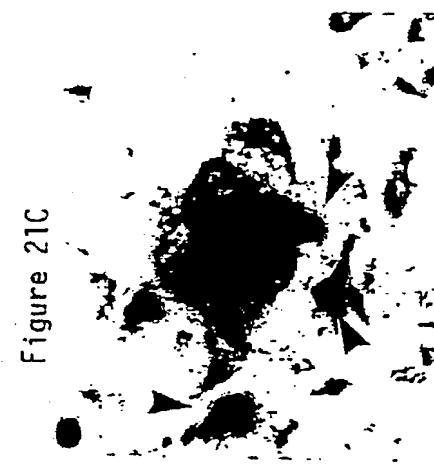


Figure 21B



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Figure 21D



Figure 21E



Figure 21F



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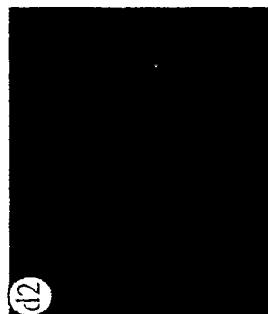


Figure 22A

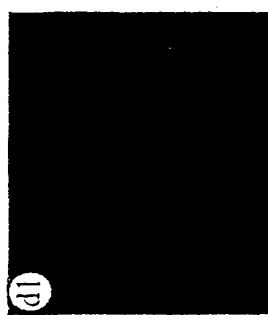


Figure 22B

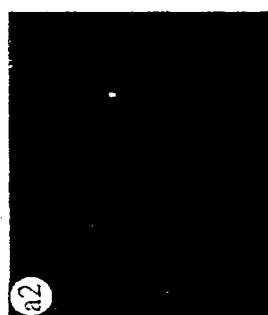


Figure 22C



Figure 22D

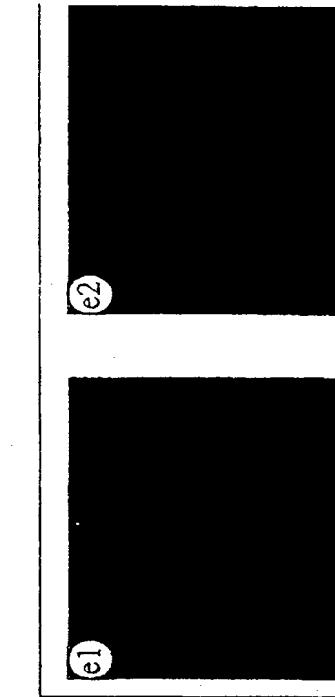


Figure 22E

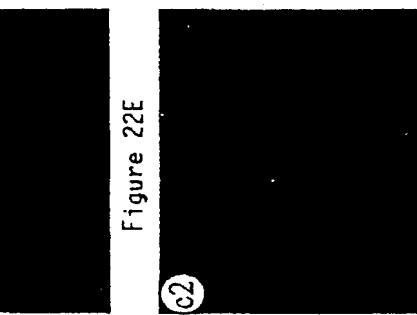


Figure 22F

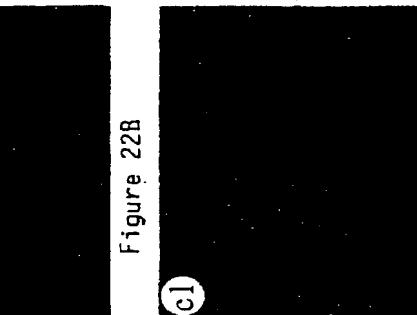


Figure 22G

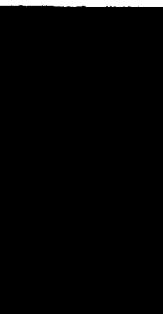


Figure 22H

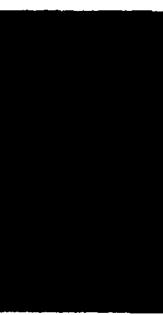


Figure 22I



Figure 22J

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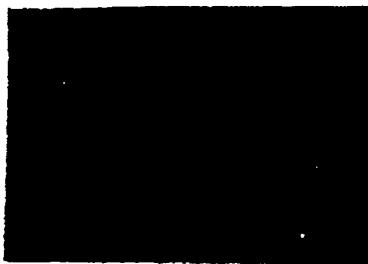


Figure 23A



Figure 23D

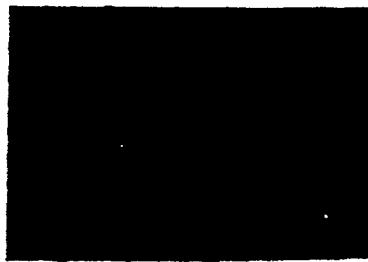


Figure 23B



Figure 23E



Figure 23C



Figure 23F

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- The attached application, or
 Application No. 10/088,138, filed on 15 March 2002,
 as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

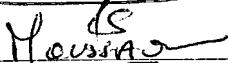
I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

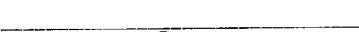
FULL NAME OF INVENTOR(S)

Inventor one: Saliha Moussaoui-Mrabet

Signature: 

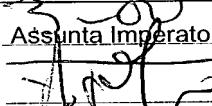
Citizen of: Morocco

Inventor two: Veronique Blanchard-Bregeon

Signature: 

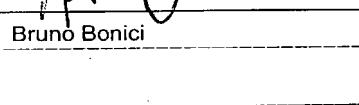
Citizen of: France

Inventor three: Assunta Imperato

Signature: 

Citizen of: Italy

Inventor four: Bruno Bonici

Signature: 

Citizen of: France

Additional inventors are being named on 1 additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

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**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- The attached application, or
 Application No. 10/088,138, filed on 15 March 2002,
 as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

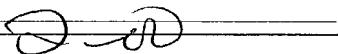
I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Salihia Moussaoui-Mrabet

Signature:  Citizen of: Morocco

Inventor two: Veronique Blanchard-Bregeon

Signature: Veronique Blanchard 10/16/02 Citizen of: France

Inventor three: Assunta Imperato

Signature:  Citizen of: Italy

Inventor four: Bruno Bonci

Signature: Bruno Bonci 16/10/02 Citizen of: France

Additional inventors are being named on 1 additional form(s) attached hereto.

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I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

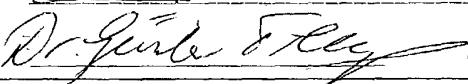
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All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor five: Gunter Tremp

Signature:  Citizen of: Germany

Inventor six: Christian Czech

Signature: _____ Citizen of: France

Additional inventors are being named on _____ additional form(s) attached hereto.

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- The attached application, or
 Application No. 10/088,138, filed on 15 March 2002,
 as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor five: Gunter Tremp

Signature: G. Tremp Citizen of: Germany

Inventor six: Christian Czech

Signature: 01/14/02 Christian Czech Citizen of: France

Additional inventors are being named on _____ additional form(s) attached hereto.

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